



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/12, 15/70, C07K 13/00, C12N 1/21, C12P 21/08, G01N 33/53, C12Q 1/68, 1/00, A61K 48/00, 37/02		A1	(11) International Publication Number: WO 95/00642 (43) International Publication Date: 5 January 1995 (05.01.95)
(21) International Application Number: PCT/US94/07089 (22) International Filing Date: 22 June 1994 (22.06.94) (30) Priority Data: 08/081,448 22 June 1993 (22.06.93) US		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TI, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.	
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(54) Title: VERTEBRATE APOPTOSIS GENE: COMPOSITIONS AND METHODS

(57) Abstract

The invention relates generally to compositions of and methods for obtaining and using a polypeptide other than BCL-2 (SEQ ID NO.5) that affects programmed vertebrate cell death. The invention relates as well to polynucleotides encoding those polypeptides, recombinant vectors carrying those sequences, the recombinant host cells including either the sequences or vectors, and recombinant polypeptides. The invention further provides methods for using the isolated, recombinant polypeptides in assays designed to select and improve substances capable of altering programmed cell death for use in diagnostic, drug design and therapeutic applications.

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bcl-x_L MSQS
bcl-x_S MSQS

bcl-x_L NRRELVVDPLS YKLSQKGYSW SQFSDVVEENR TEAPEGTESE METPSAINGN
bcl-x_S NRRELVVDPLS YKLSQKGYSW SQFSDVVEENR TEAPEGTESE METPSAINGN

bcl-x_L PSWHLADSPA VNGATAHSSS LDAREVIPMA AVKQALREA GDEFELRYRR
bcl-x_S PSWHLADSPA VNGATAHSSS LDAREVIPMA AVKQALREA GDEFELRYRR

bcl-x_L AFSDLTSQLH ITPGTAYQSF EQVNVNELFRD GVNWGRIVAF PSFGGALCWE
bcl-x_S AFSDLTSQLH ITPGTAYQSF EQ.....
bcl-x_I VVNELFRD GVNWGRIVAF PSFGGALCWE

bcl-x_L SVDKEMQVLV SRIAAMMAYT LNDHLEPWIQ ENGGWDTFVE LYGNMAAAES
bcl-x_S DTFVE LYGNMAAAES
bcl-x_I SVDKEMQVLV SRIAAMMAYT LNDHLEPWIQ ENGGW

bcl-x_L RKGQERFNWR FLTGTNTVAGV VLLGSLFSRK
bcl-x_S RKGQERFNWR FLTGTNTVAGV VLLGSLFSRK

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DescriptionVERTEBRATE APOPTOSIS GENE: COMPOSITIONS AND METHODSTechnical Field of the Invention

This invention relates generally to compositions of and methods for altering or regulating programmed vertebrate cell death (apoptosis). The invention relates more particularly to DNA sequences encoding polypeptides that promote or inhibit apoptosis, recombinant vectors carrying those sequences, the recombinant host cells including either the sequences or vectors, and polypeptides. The invention includes as well methods for using the isolated, recombinant polypeptides in assays designed to select and improve among candidate substances that affect apoptosis and polypeptides and polynucleotides for use in diagnostic, drug design and therapeutic applications.

Background of the Invention

The control of cell number in multicellular eukaryotes represents a balance between cell proliferation and cell death. Although a great deal has been learned in recent years about the regulation of cell proliferation, relatively little is known about the regulation of cell death (Ellis et al., 1991; Raff, 1992). Recently, attention has begun to focus on the mechanisms that regulate programmed cell death (apoptosis) (Williams, 1991). Apoptosis is an active process by which many cells die during development and self-maintenance in complex eukaryotes (Kerr et al., 1972). Cell death by apoptosis occurs when a cell activates an internally encoded suicide program as a result of either extrinsic or intrinsic signals. Apoptotic cell death is characterized by plasma membrane blebbing, cell volume loss, nuclear condensation, and endonucleolytic degradation of DNA at nucleosomal intervals (Wyllie et al., 1980).

Two of the best studied vertebrate systems in which programmed cell death plays a role are neural and lymphoid development. During T cell development in the thymus, each individual T cell precursor generates a unique T cell antigen receptor (TCR) by combinatorial rearrangement of TCR gene segments and the cell subsequently undergoes a series of selection processes (Blackman et al., 1990; Rothenberg, 1992). T cells expressing autoreactive TCRs are deleted by apoptosis as a result of negative selection (Murphy et al., 1990). Other cells undergo positive selection through interaction with self-encoded major histocompatibility complex (MHC) molecules expressed on thymic stromal cells, a process which prevents programmed cell death and results in the subsequent MHC-restriction of the mature T cell repertoire. An additional set of thymic cells die as a result of neglect, the absence of either negative or positive selection. Extensive cell death also occurs in the developing nervous system (Cowan et al., 1984; Davies, 1987; Oppenheim, 1991). Following an initial expansion of neurons during development, a significant reshaping of neural structures occurs as a result of the establishment of synaptic interactions. During this reshaping period, the survival of neurons is determined by their supply of neurotrophic growth factors. Cells that become growth-factor deficient die by apoptosis. Once synaptic connections are established, the surviving neurons develop into post-mitotic cells with extended life spans. Thus, programmed cell death plays an essential role in lymphoid development by removing autoreactive T cells and within the nervous system by facilitating the establishment of effective synaptic networks.

Because of the importance of programmed cell death to these developmental processes, considerable interest has arisen in genes that are capable of regulating apoptosis. One of the most important advances in the understanding of the regulation of apoptotic cell death in vertebrates has come from studies of the oncogene bcl-2. bcl-2 was originally cloned from the breakpoint of a t(14;18) translocation present in

many human B cell lymphomas (Cleary et al., 1986; Tsujimoto et al., 1986). This translocation results in the deregulated expression of the bcl-2 gene as result of its juxtaposition with the immunoglobulin heavy chain gene locus (Bakhshi et al., 1985). In vitro, BCL-2 (the gene product of bcl-2; SEQ ID NO:5) has been shown to prevent apoptotic cell death in cultured cells which are deprived of growth factors (Vaux et al., 1988; Hockenberry et al., 1990; Nuñez et al., 1990; Borzillo et al., 1992; Garcia et al., 1992). However, BCL-2 is not able to block apoptosis in all cells induced by cytokine deprivation or receptor-mediated signalling. For example, BCL-2 prevents apoptosis in hematopoietic cell lines dependent on certain interleukins (IL) IL-3, IL-4, or GM-CSF but it fails to prevent other cell lines from apoptosis following IL-2 or IL-6 deprivation (Nuñez et al., 1990). Overexpression of BCL-2 also fails to prevent antigen receptor-induced apoptosis in some B cell lines (Cuende et al., 1993). In vivo, BCL-2 prevents many, but not all, forms of apoptotic cell death that occur during lymphoid (Sentman et al., 1991; Strasser et al., 1991a; Strasser et al., 1991b; Seigel et al., 1992) and neural (Allsop et al., 1993) development. Expression of a bcl-2 transgene can prevent radiation- and calcium ionophore-induced apoptotic cell death in thymocytes, but does not inhibit the process of negative selection (Sentman et al., 1991; Strasser et al., 1991a). Similarly, overexpression of bcl-2 can prevent apoptosis in neurons dependent on nerve growth factor, but not neurons dependent upon ciliary neurotrophic factor. (Allsop et al., 1993) These results suggest the existence of multiple independent intracellular mechanisms of apoptosis, some of which can be prevented by BCL-2 and others which are unaffected by this gene. Alternatively, these additional pathways may involve proteins that differentially regulate BCL-2 function.

Brief Summary of the Invention

In one aspect, the present invention provides an isolated and purified polynucleotide that encodes a polypeptide other than BCL-2 (SEQ

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ID NO:5) that promotes or inhibits programmed vertebrate cell death. In a preferred embodiment, a polynucleotide of the present invention is a DNA molecule from a vertebrate species. A preferred vertebrate is a mammal. A preferred mammal is a human. More preferably, a polynucleotide of the present invention encodes polypeptides designated BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) and BCL-X_I (SEQ ID NO:4). Even more preferred, a polynucleotide of the present invention encodes a polypeptide comprising the amino acid residue sequence of SEQ ID NO:2 (Figure 1) or SEQ ID NOS:4, 7, and 9 (Figures 4A - 4C). Most preferably, an isolated and purified polynucleotide of the invention comprises the nucleotide base sequences of SEQ ID NO: 1 and 3 (Figure 1A); SEQ ID NO:6 (Figure 4A); SEQ ID NO:8 (Figure 4B) or their homologues from other vertebrate species.

Yet another aspect of the present invention contemplates an isolated and purified polynucleotide comprising a base sequence that is identical or complementary to a segment of at least 10 contiguous bases of SEQ ID NOS:1 and 3 (Figure 1A), wherein the polynucleotide hybridizes to a polynucleotide that encodes a polypeptide other than BCL-2 that promotes or inhibits programmed vertebrate cell death. Preferably, the isolated and purified polynucleotide comprises a base sequence that is identical or complementary to a segment of at least 25 to 70 contiguous bases of SEQ ID NOS: 1 and 3 (Figure 1A). For example, a polynucleotide of the invention can comprise a segment of bases identical or complementary to 40 or 55 contiguous bases of the disclosed nucleotide sequences.

In another embodiment, the present invention contemplates an isolated and purified polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death. Preferably, a polypeptide of the invention is a recombinant polypeptide. More preferably, a polypeptide of the present invention is BCL-X_L (SEQ ID

NO:7), BCL-X_s (SEQ ID NO:9) and BCL-X₁ (SEQ ID NO:4). Even more preferably, a polypeptide of the present invention comprises the amino acid residue sequence of SEQ ID NO:2 (Figure 1A) or Figures 4A-4C (SEQ ID NOS: 4, 7, and 9).

In an alternative embodiment, the present invention provides an expression vector comprising a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death. Preferably, an expression vector of the present invention comprises a polynucleotide that encodes BCL-X_L (SEQ ID NO:6), BCL-X_s (SEQ ID NO:9) and BCL-X₁ (position 510-698 of SEQ ID NO:6). More preferably an expression vector of the present invention comprises a polynucleotide that encodes a polypeptide comprising the amino acid residue sequence of SEQ ID NO:2 (Figure 1A) or SEQ ID NOS:4, 7 and 9 (Figures 4A-4C). More preferably, an expression vector of the present invention comprises a polynucleotide comprising the nucleotide base sequence of SEQ ID NOS:1 and 3 (Figure 1A) or SEQ ID NOS:6 and 8 (Figures 4A-4C). Even more preferably, an expression vector of the invention comprises a polynucleotide operatively linked to an enhancer-promoter. More preferably still, an expression vector of the invention comprises a polynucleotide operatively linked to a prokaryotic promoter. Alternatively, an expression vector of the present invention comprises a polynucleotide operatively linked to an enhancer-promoter that is a eukaryotic promoter, and the expression vector further comprises a polyadenylation signal that is positioned 3' of the carboxy-terminal amino acid and within a transcriptional unit of the encoded polypeptide.

In yet another embodiment, the present invention provides a recombinant host cell transfected with a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death. Figures 1 and 4 set forth nucleotide and amino acid sequences from the exemplary vertebrates chicken and

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human. Also contemplated by the present invention are homologous or biologically equivalent polynucleotides and polypeptides other than BCL-2 (SEQ ID NO:5) found in other vertebrates. Preferably, a recombinant host cell of the present invention is transfected with the polynucleotide that encodes BCL-X_L (SEQ ID NO:6), BCL-X_S (SEQ ID NO:8) and BCL-X_I (position 510 to 698 of SEQ ID NO:6). More preferably, a recombinant host cell of the present invention is transfected with the polynucleotide sequence of SEQ ID NOS:1 and 3 (Figure 1A) or SEQ ID NOS:6 and 8 (Figures 4A-4C). Even more preferably, a host cell of the invention is a eukaryotic host cell. Still more preferably, a recombinant host cell of the present invention is a vertebrate cell. Preferably, a recombinant host cell of the invention is a mammalian cell.

In another aspect, a recombinant host cell of the present invention is a prokaryotic host cell. Preferably, a recombinant host cell of the invention is a bacterial cell, preferably a strain of *Escherichia coli*. More preferably, a recombinant host cell comprises a polynucleotide under the transcriptional control of regulatory signals functional in the recombinant host cell, wherein the regulatory signals appropriately control expression of a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death in a manner to enable all necessary transcriptional and post-transcriptional modification.

In yet another embodiment, the present invention contemplates a process of preparing a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death comprising transfecting a cell with polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death to produce a transformed host cell; and maintaining the transformed host cell under biological conditions sufficient for expression of the polypeptide. Preferably, the transformed host cell is a eukaryotic cell. More preferably still, the eukaryotic cell is a vertebrate

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cell. Alternatively, the host cell is a prokaryotic cell. More preferably, the prokaryotic cell is a bacterial cell of the DH5 α strain of *Escherichia coli*. Even more preferably, a polynucleotide transfected into the transformed cell comprises the nucleotide base sequence of SEQ ID NOS:1 and 3 (Figure 1A) or SEQ ID NOS:6 and 8 (Figures 4A-4C). Figures 1 and 4 set forth nucleotide and amino acid sequences for the exemplary vertebrates chicken and human. Also contemplated by the present invention are homologues or biologically equivalent polynucleotides and polypeptides other than bcl-2/BCL-2 (SEQ ID NO:5) found in other vertebrates.

In still another embodiment, the present invention provides an antibody immunoreactive with a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death. Figures 1 and 4 set forth nucleotide and amino acid sequences from the exemplary vertebrates chicken and human. Also contemplated by the present invention are antibodies immunoreactive with homologues or biologically equivalent polynucleotides and polypeptides other than bcl-2 found in other vertebrates. Preferably, an antibody of the invention is a monoclonal antibody. More preferably, a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death is BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9), or BCL-X_I (SEQ ID NO:4). Even more preferably, a polypeptide comprises the amino acid residue sequence of SEQ ID NO:2 (Figure 1) or SEQ ID NOS: 4, 7, and 9 (Figures 4A-4C).

In another aspect, the present invention contemplates a process of producing an antibody immunoreactive with a polypeptide other than BCL-2 that promotes or inhibits programmed vertebrate cell death comprising the steps of (a) transfecting a recombinant host cell with a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death; (b) culturing the host cell under conditions sufficient for expression of the

polypeptide; (c) recovering the polypeptide; and (d) preparing the antibody to the polypeptide. Figures 1 and 4 set forth nucleotide and amino acid sequences from the exemplary vertebrates chicken and human.

Preferably, the host cell is transfected with the polynucleotide of SEQ ID NOS:1 and 3 (Figure 1A) or SEQ ID NOS:6 and 8 (Figures 4A-4C). Even more preferably, the present invention provides an antibody prepared according to the process described above. Also contemplated by the present invention is the use of homologues or biologically equivalent polynucleotides and polypeptides other than BCL-2 (SEQ ID NO:5) found in other vertebrates to produce antibodies.

Alternatively, the present invention provides a process of detecting a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death, wherein the process comprises immunoreacting the polypeptide with an antibody prepared according to the process described above to form an antibody-polypeptide conjugate, and detecting the conjugate.

In yet another embodiment, the present invention contemplates a process of detecting a messenger RNA transcript that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death, wherein the process comprises (a) hybridizing the messenger RNA transcript with a polynucleotide sequence that encodes that polypeptide to form a duplex; and (b) detecting the duplex. Alternatively, the present invention provides a process of detecting a DNA molecule that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death, wherein the process comprises (a) hybridizing DNA molecules with a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death to form a duplex; and (b) detecting the duplex.

In another aspect, the present invention contemplates a diagnostic assay kit for detecting the presence of a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death in a biological sample, where the kit comprises a first container containing a first antibody capable of immunoreacting with a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death, with the first antibody present in an amount sufficient to perform at least one assay. Preferably, an assay kit of the invention further comprises a second container containing a second antibody that immunoreacts with the first antibody. More preferably, the antibodies used in an assay kit of the present invention are monoclonal antibodies. Even more preferably, the first antibody is affixed to a solid support. More preferably still, the first and second antibodies comprise an indicator, and, preferably, the indicator is a radioactive label or an enzyme.

In an alternative aspect, the present invention provides a diagnostic assay kit for detecting the presence, in biological samples, of a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death, the kits comprising a first container that contains a second polynucleotide identical or complementary to a segment of at least 10 contiguous nucleotide bases of bcl-x_L (SEQ ID NO:6), bcl-x_S (SEQ ID NO:8), or bcl-x_I (position 510 to 698 of SEQ ID NO:6).

In another embodiment, the present invention contemplates a diagnostic assay kit for detecting the presence, in a biological sample, of an antibody immunoreactive with a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death, the kit comprising a first container containing a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death

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that immunoreacts with the antibody, with the polypeptide present in an amount sufficient to perform at least one assay.

In another aspect, the present invention provides a method of preventing or treating programmed cell death in cells, the method comprising:

- (a) preparing a non-pathogenic vector comprising the a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death; and
- (b) introducing the non-pathogenic vector into cells undergoing or likely to undergo programmed cell death.

In a preferred embodiment, the vector comprises a retrovirus, a vaccinia virus, a picornavirus, a coronavirus, a togavirus, or a rhabdovirus altered in such a way as to render it non-pathogenic.

Preferably, the cell is a neuronal cell and the method further comprises introducing the vector into the cells undergoing or likely to undergo programmed cell death by a process comprising transplanting cells of a multipotent neural cell line into a region of the central nervous system in which said neuronal cells undergoing or likely to undergo programmed cell death are located. Alternatively, the vector is introduced into neuronal cells of an animal by injection of the vector at the site of the peripheral nerve endings of the neuronal cells undergoing or likely to undergo cell death or into neuronal cells in culture likely to undergo or undergoing cell death by incubation of the vector with the neuronal cells.

In another aspect, the present invention provides a method of preventing or treating programmed cell death in neuronal cells, the method comprising:

- (a) preparing a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death;

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- (b) combining the polypeptide with a physiologically acceptable carrier to form a pharmaceutical composition; and
- (c) administering the composition to neurons likely to undergo or undergoing programmed cell death.

In yet another aspect, the present invention provides a method of delivering a gene that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death for gene therapy, the method comprising:

- (a) providing the vector of claim 10;
- (b) combining the vector with a physiologically acceptable carrier to form a pharmaceutical composition; and
- (c) administering said pharmaceutical composition so that the vector will reach the intended cell targets.

In a preferred embodiment, the pharmaceutical composition is introduced by injection into an animal at the site of said cell targets and the cell targets are in the central nervous system and the pharmaceutical composition is introduced by injection into an animal at the site of the peripheral nerve ending which originate from neurons located at the site of said cell targets.

In still yet another aspect, the present invention provides a method of treating tumorogenic diseases, the method comprising:

- (a) providing an expression vector according to claim 10;
- (b) combining the vector with a physiologically acceptable carrier to form a pharmaceutical composition; and
- (c) administering the composition to tumor cell targets.

Brief Description of the Drawings

In the drawings, which form a portion of the specification:

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Figure 1. Nucleotide sequence and predicted open reading frame of chicken bcl-x (SEQ ID NOS:1 and 3). A. The nucleotide sequence of chicken bcl-x (SEQ ID NOS:1 and 3) represents a composite sequence derived from a cDNA clone and the corresponding genomic clone. The cDNA consisted of a 1.3 kb clone whose 5' end is indicated by the arrow. The 5' end of the sequence has been obtained from a genomic clone and shows the 5' end of a predicted open reading frame as well as 257 additional nucleotides ending with a 5' NarI site. The putative initiation codon conforms poorly to the consensus eukaryotic translation initiation sequence while a consensus eukaryotic initiation sequence appears out-of-frame 32 nucleotides 5' of this site. Both the cDNA and genomic sequences end at a natural EcoRI site. B. The amino acid alignment of the predicted open reading frame from chicken bcl-x (SEQ ID NO:2) (upper sequence) to the open reading frame from the human bcl-2b (SEQ ID NO:5) protein sequence (lower sequence). A search of GenBank revealed that bcl-x displayed significant homology with all forms of bcl-2 present in GenBank with highest homology to the bcl-2b form. Like the bcl-2b cDNA, it appears that the bcl-x cDNA (SEQ ID NOS: 1 and 3) arose from an unspliced RNA since it is colinear with the genomic sequence from which it is derived. Figure 2 contains two panels.

Figure 2. bcl-x mRNA expression in tissues isolated from a newly hatched chicken. Tissue mRNAs isolated from a chicken on the day of hatching were hybridized with a chicken bcl-x-specific probe as well as a murine bcl-2 probe. While the murine bcl-2 probe recognized a 6.5 kb mRNA indicated by the arrow that was present in all tissues tested, the bcl-x probe hybridized to a 2.7 kb mRNA indicated by the arrow.

Figure 3. Southern blot analysis of bcl-x and bcl-2 using chicken, mouse, and human genomic DNA. Genomic DNA from chickens, mice, and humans were digested with BamHI (B), HindIII (H), and PstI (P). The resulting DNA was separated by gel electrophoresis, and then transferred

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to nitrocellulose. The Southern blots were hybridized with specific probes isolated from the first coding exon of murine bcl-2 and a similar region from chicken bcl-x, and the resulting autoradiograms are shown.

Figure 4. Predicted amino acid sequence of human mRNAs related to chicken bcl-x (SEQ ID NO:2). In Figures 4A and 4B are the predicted open reading frames of two distinct human cDNAs (bcl-x_L (SEQ ID NO:6) and bcl-x_S (SEQ ID NO:8), respectively) with homology to chicken bcl-x. In Figure 4C, the 63 amino acid region (SEQ ID NO:4) of human BCL-X_L (SEQ ID NO:7) deleted in human BCL-X_S (SEQ ID NO:9) is denoted by dots. A predicted 19 amino acid hydrophobic domain and flanking charged residues which are present in both BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) are indicated by underlining and asterisks respectively. The average hydrophobicity of this domain which is present in both BCL-X_L (SEQ ID NO:7) and BCL-X_S (SEQ ID NO:9), is 1.3 as calculated by the method of Kyte-Doolittle. Figure 4 contains three panels.

Figure 5. Translational products of bcl-x_L and bcl-x_S mRNAs. Both bcl-x_L and bcl-x_S mRNAs were subjected to in vitro translation in the presence of 35S-radiolabeled methionine. The resulting translated proteins were run on an SDS-polyacrylamide gel. Sizes of the resulting proteins are indicated on the right in kilodaltons. The result of a translation reaction using bcl-x_L antisense mRNA (bcl-x_L-as) is shown as a control to demonstrate the specificity of the translational products.

Figure 6. The effect of bcl-x_L expression on FL5.12 cell survival following IL-3 withdrawal. Stable transfectants of FL5.12 with the pSFFV-Neo vector containing bcl-x_L in the forward (bcl-x_L; B) and reverse orientations (bcl-x_Lrev; N), bcl-2 (H), bcl-2 + bcl-x_L (F), and vector control (Neo; J) were prepared as described in Examples. Cell survival was determined by trypan blue exclusion at the indicated time points. Data is presented as the mean + S.D. of triplicate cultures.

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Figure 7. Stable expression of bcl-x_s prevents bcl-2-induced survival of FL5.12 cells upon IL-3 withdrawal. A. Stable transfectants of FL5.12 expressing bcl-2 (J), bcl-x_s (H), bcl-2 + bcl-x_s (E), or the selectable marker neomycin (Neo; B) alone were prepared as described in Examples. In addition, individual subclones of bcl-x_s (bcl-x_s Clone 1 [N] and bcl-x_s Clone 2 [F]) were analyzed. At time zero, exponentially growing cells were withdrawn from IL-3 support, and survival analyzed over time by trypan blue exclusion. The lower portion of the figure shows the flow cytometry analysis of the neomycin (Neo), bcl-2, and bcl-2 + bcl-x_s bulk populations. Cells were permeabilized as indicated in the Examples, and then stained with a monoclonal antibody specific for human bcl-2 (thick line) or an irrelevant control antibody (thin line). B. Survival of individual bcl-2 + bcl-x_s subclones following IL-3 withdrawal. The survival of subclones expressing both bcl-2 and bcl-x_s were analyzed following growth factor withdrawal as described above (bcl-2 + bcl-x_s Clone 1 and bcl-2 + bcl-x_s Clone 2). In the lower half of the figure, flow cytometry analysis for bcl-2 expression in the neomycin, bcl-2, bcl-2 + bcl-x_s Clone 1, bcl-2 + bcl-x_s Clone 2 populations. Cells were permeabilized as described in the Examples and then stained with a monoclonal antibody specific for human bcl-2 (thick line) or a irrelevant control isotype-matched antibody (thin line), and the data displayed as fluorescence intensity versus cell number. C. Expression of bcl-x RNA in stably transfected FL5.12 cell lines. RNA was isolated from each of the clones indicated above and analyzed on a Northern blot by hybridization with a bcl-x-specific and b-actin-specific probes. Figure 7 consists of three panels.

Figure 8. bcl-2-induced survival of FL5.12 cells following IL-3 withdrawal is unaffected by antisense bcl-x_s expression. FL5.12 cells stably transfected with either bcl-2 (J) or bcl-2 plus an expression vector containing bcl-x_s cloned in the reverse orientation (bcl-2 + bcl-x_srev; H), or neomycin (Neo; B) alone were analyzed for survival following IL-3

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withdrawal. In the lower panels, the level of bcl-2 expression is analyzed on a fluorescence-activated cell sorter by staining permeabilized cells with monoclonal antibodies specific for bcl-2 (thick lines) or an irrelevant control antibody (thin lines).

Figure 9. Expression of bcl-x in human thymocytes and T cells. To examine the expression of bcl-x during T cell development, RNA was prepared from unseparated thymocytes, immature thymocytes, mature thymocytes, and peripheral blood T cells as described in the Examples. The immature thymocytes, mature thymocytes, and T cell populations were further analyzed by stimulation in vitro with PMA and ionomycin for 6 to 8 hours in complete media. Resulting RNAs were isolated by the guanidinium isothiocyanate method and subjected to Northern blot analysis. Top panels demonstrate the equalization of the RNA samples used for analysis, and the lower two panels represent hybridization with a bcl-x-specific probe or an HLA class I-specific probe.

Figure 10. Pattern of bcl-x and bcl-2 mRNA induction following peripheral blood T cell activation. Peripheral blood T cells were isolated as described in Examples and then subjected to activation with a combination of PMA and ionomycin for 0, 6, 12, or 24 hours as indicated. The relative induction of bcl-x and bcl-2 was analyzed by equalizing RNA from the different time points for ribosomal RNA (upper panel). Duplicate Northern blots were probed for either bcl-x or bcl-2, and HLA class I mRNA. In the data shown, the bcl-x autoradiogram has been exposed for 8 hours, while the bcl-2 autoradiogram has been exposed for 15 days; both probes were of similar length and base composition.

Figure 11. Analysis of relative proportions of bcl-x_s and bcl-x_L mRNAs expressed in human thymocytes, T cells, and adult brain. PCR primers that flank the 5' and 3' ends of the open reading frame of bcl-x_s were used to amplify bcl-x_L and bcl-x_s simultaneously. Using these

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primers, RNAs from various sources were subjected to PCR analysis. When a bcl-x_s template is utilized, a single band of 591 base pairs is produced, whereas when a bcl-x_L template is used a single band of 780 base pairs is observed. Molecular weight markers from HaeIII-digested fX 174 are indicated (M). A. Lanes represent products from PCR reactions using a bcl-x_s template, a bcl-x_L template, and using RNA from unstimulated peripheral blood T cells, peripheral blood T cells stimulated for 6 hours with PMA and ionomycin, unseparated human thymocytes, and from adult brain. The identification of the observed bands in the tissue samples as bcl-x_L and bcl-x_s has been verified by cloning and partial sequencing of PCR products of reverse transcribed RNA from each of the tissue sources. B. A titration curve to demonstrate the validity of the PCR assay in quantitating the relative ratios of bcl-x_L and bcl-x_s. The figure depicts the products of PCR reactions separated on 1% agarose gels and stained with ethidium bromide. The PCR was performed using a ratio of bcl-x_L to bcl-x_s that varied from 0:10 to 10:0 in unit increments. The resulting products reflect the relative proportions of bcl-x_L and bcl-x_s template added to the reaction mix.

Detailed Description of the Invention

I. The Invention

The present invention provides DNA segments, purified polypeptides, methods for obtaining antibodies, methods of cloning and using recombinant host cells necessary to obtain and use recombinant apoptosis polypeptides. Accordingly, the present invention concerns generally compositions and methods for the preparation and use of polypeptides other than BCL-2 (SEQ ID NO:5) that promote or inhibit programmed vertebrate cell death.

II. Polynucleotide

A. Isolated and purified polynucleotide that encode polypeptides other than BCL-2 that promote or inhibit programmed vertebrate cell death.

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In one aspect, the present invention provides an isolated and purified polynucleotide that encodes a polypeptide other than BCL-2 that promotes or inhibits programmed vertebrate cell death. In a preferred embodiment, a polynucleotide of the present invention is a DNA molecule from a vertebrate species. A preferred vertebrate is a mammal. A preferred mammal is a human. In a preferred embodiment, a polynucleotide of the present invention is a DNA molecule. More preferably, a polynucleotide of the present invention encodes polypeptides designated BCL-X₁ (SEQ ID NO:6), BCL-x₃ (SEQ ID NO:8) and BCL-X₁ (SEQ ID NO:4). Even more preferred, a polynucleotide of the present invention encodes a polypeptide comprising the amino acid residue sequences of SEQ ID NOS:2, 4, 7, and 9. Most preferably, an isolated and purified polynucleotide of the invention comprises the nucleotide base sequences of SEQ ID NOS:1, 3, 6, and 8. In a preferred embodiment, a polynucleotide is bcl-x (SEQ ID NOS:1 and 3).

As used herein, the term "polynucleotide" means a sequence of nucleotides connected by phosphodiester linkages. Polynucleotides are presented herein in the direction from the 5' to the 3' direction. A polynucleotide of the present invention can comprise from about 680 to about several hundred thousand base pairs. Preferably, a polynucleotide comprises from about 680 to about 150,000 base pairs. Preferred lengths of particular polynucleotides are set forth hereinafter. As used herein, polynucleotides (e.g., genes) are designated using lower case letters (e.g., bcl-2, bcl-x).

A polynucleotide of the present invention can be a deoxyribonucleic acid (DNA) molecule or ribonucleic acid (RNA) molecule. Where a polynucleotide is a DNA molecule, that molecule can be a gene or a cDNA molecule. Nucleotide bases are indicated herein by a single letter code: adenine (A), guanine (G), thymine (T), cytosine (C), inosine (I) and uracil (U).

A polynucleotide of the present invention can be prepared using standard techniques well known to one of skill in the art. The preparation of a cDNA molecule encoding a polypeptide other than BCL-2 that promotes or inhibits programmed vertebrate cell death of the present invention is described hereinafter in Examples 1 and 3. A polynucleotide can also be prepared from genomic DNA libraries using lambda phage technologies.

In another aspect, the present invention provides an isolated and purified polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death, where the polynucleotide is preparable by a process comprising the steps of constructing a library of cDNA clones from a cell that expresses the polypeptide; screening the library with a labelled cDNA probe prepared from RNA that encodes the polypeptide; and selecting a clone that hybridizes to the probe. Preferably, the polynucleotide of the invention is prepared by the above process. More preferably, the polynucleotide of the invention encodes a polypeptide that has the amino acid residue sequence of SEQ ID NOS:6 and 8. More preferably still, the polynucleotide comprises the nucleotide sequence of SEQ ID NOS: 1 and 3.

In an initial series of studies the inventors used low stringency hybridization with a murine bcl-2 cDNA probe to identify bcl-2-related genes in chicken lymphoid cells. One of the isolated clones, bcl-x, contained an open reading frame which displayed 44% amino acid identity with human or mouse BCL-2. Southern blotting revealed that chicken BCL-X is encoded by a gene that is distinct from chicken bcl-2. Chicken bcl-x was subsequently used to isolate two distinct cDNAs derived from the human bcl-x gene. These two cDNAs differ in their predicted open reading frames. One cDNA, bcl-x_L (SEQ ID NO:6), contains an open reading frame with 233 amino acids (SEQ ID NO:7) with

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similar domains to those previously described for BCL-2 (SEQ ID NO:5). The other cDNA, bcl-x_s (SEQ ID NO:8), encodes a 170 amino acid protein (SEQ ID NO:9) in which the region of highest homology to BCL-2 (SEQ ID NO:5) has been deleted. The difference in these two cDNAs arises from differential usage of two 5' splice sites within the first coding exon. When the ability of these two proteins to regulate apoptotic cell death was compared, it was found that BCL-X_L rendered cells resistant to apoptotic cell death upon growth factor deprivation, while BCL-X_s could prevent overexpression of bcl-2 from inducing resistance to apoptotic cell death. Thus, it appears that the regulation of both expression and splicing of bcl-x during development may play a critical role in determining the susceptibility of cells to programmed cell death. Consistent with this observation, the inventors have found that immature thymocytes which are in the process of undergoing selection in the thymus express a high level of bcl-x_s message. The expression of bcl-x_s likely accounts for the inability of bcl-2 to prevent death by negative selection in this cell population. Bcl-x_s can function as a dominant regulator of cell death even in the presence of high level bcl-2 expression. In addition, the inventors have found that mature neural structures constitutively express only the bcl-x_L mRNA. Thus, BCL-X_L may contribute to the resistance to programmed cell death and long term viability of this important post-mitotic cell population. Together, the present studies indicate that the two bcl-x gene products may regulate one or more BCL-2-independent pathways of apoptotic cell death.

The bcl-x gene has been highly conserved in vertebrate evolution and bcl-x mRNA is expressed in a variety of tissues with the highest levels of mRNA observed in the lymphoid and central nervous systems. The present inventors have isolated two distinct bcl-x mRNA species from human tissues. These two cDNAs result from the alternative use of two distinct 5' splice sites located within the first coding exon of the bcl-x gene. The longer cDNA, bcl-x_L (SEQ ID NO:6), encodes a protein (SEQ ID

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NO:7) that appears to be similar in size and predicted structure to BCL-2. The shorter cDNA, bcl-x_s (SEQ ID NO:8), contains a deletion of the 63 amino acids (SEQ ID NO:4) from the bcl-x_L (SEQ ID NO:7) open reading frame that constitutes the region of highest amino acid identity between BCL-X_L (SEQ ID NO:7) and BCL-X_s (SEQ ID NO:9).

B. Probes and Primers.

In another aspect, DNA sequence information provided by the present invention allows for the preparation of relatively short DNA (or RNA) sequences having the ability to specifically hybridize to gene sequences of a selected polynucleotide disclosed herein. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of a selected nucleotide sequences of SEQ ID NOS:1, 3, 6, and 8. Such nucleic acid probes specifically hybridize to a polynucleotide encoding a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death. Most importantly, the probes can be used in a variety of assays for detecting the presence of complementary sequences in a given sample.

In certain embodiments, it is advantageous to use oligonucleotide primers. The sequence of such primers is designed using a polynucleotide of the present invention for use in detecting, amplifying or mutating a defined segment of a gene or polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death from cells using PCR technology.

To provide certain of the advantages in accordance with the present invention, a preferred nucleic acid sequence employed for hybridization studies or assays includes probe molecules that are complementary to at least a 10 to 70 or so long nucleotide stretch of a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death, such

as that shown in SEQ ID NOS: 2, 4, 7, and 9 (Figures 1 and 4A-4C). A size of at least 10 nucleotides in length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 10 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained, one will generally prefer to design nucleic acid molecules having gene-complementary stretches of 25 to 40 nucleotides, 55 to 70 nucleotides, or even longer where desired. Such fragments can be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102, herein incorporated by reference, or by excising selected DNA fragments from recombinant plasmids containing appropriate inserts and suitable restriction enzyme sites.

In another aspect, the present invention contemplates an isolated and purified polynucleotide comprising a base sequence that is identical or complementary to a segment of at least 10 contiguous bases wherein the polynucleotide hybridizes to a polynucleotide that encodes a polypeptide other than BCL-2 (SQ ID NO:5) that promotes or inhibits programmed vertebrate cell death. Preferably, the isolated and purified polynucleotide comprises a base sequence that is identical or complementary to a segment of at least 25 to 70 contiguous bases of SEQ ID NOS:1 and 3 (Figure 1). For example, a polynucleotide of the invention can comprise a segment of bases identical or complementary to 40 or 55 contiguous bases of the disclosed nucleotide sequences.

Accordingly, a polynucleotide probe molecule of the invention can be used for its ability to selectively form duplex molecules with complementary stretches of a gene. Depending on the application envisioned, one will desire to employ varying conditions of hybridization

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to achieve varying degree of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids. For example, one will select relatively low salt and/or high temperature conditions, such as provided by 0.02 M-0.15 M NaCl at temperatures of 50°C to 70°C. Those conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death coding sequence from other cells, functional equivalents, or the like, less stringent hybridization conditions are typically needed to allow formation of the heteroduplex. In these circumstances, one can desire to employ conditions such as 0.15 M-0.9 M salt, at temperatures ranging from 20°C to 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it is advantageous to employ a polynucleotide of the present invention in combination with an appropriate label for detecting hybrid formation. A wide variety of appropriate labels are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal.

In general, it is envisioned that a hybridization probe described herein is useful both as a reagent in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions depend as is well known in the art on the particular circumstances and criteria required (e.g., on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe). Following washing of the matrix to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

II. Polypeptide other than BCL-2 that promotes or inhibits programmed cell death.

In one embodiment, the present invention contemplates an isolated and purified a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death. SEQ ID NOS: 1 and 3, 6, and 8 set forth nucleotide and SEQ ID NOS: 2, 4, 7, and 9 amino acid sequences from the exemplary vertebrates chicken and human. In a preferred embodiment, a polypeptide of the present invention is a polypeptide from a vertebrate species. A preferred vertebrate is a mammal. A preferred mammal is a human. Preferably, that polypeptide is a recombinant polypeptide. More preferably, a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death of the present invention is BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4). Upper case letters (e.g. BCL-X, BLC-2) herein to indicate polypeptides (e.g., products of gene expression). Even more preferably, a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell deaths of the present invention comprises the amino acid residue sequences of SEQ ID NOS:2, 4, 7, and 9.

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Polypeptides are disclosed herein as amino acid residue sequences. Those sequences are written left to right in the direction from the amino to the carboxy terminus. In accordance with standard nomenclature, amino acid residue sequences are denominated by either a single letter or a three letter code as indicated below.

<u>Amino Acid Residue</u>	<u>3-Letter Code</u>	<u>1-Letter Code</u>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Modifications and changes can be made in the structure of a polypeptide of the present invention and still obtain a molecule having like characteristics. For example, certain amino acids can be substituted for other amino acids in a sequence without appreciable loss of receptor

activity. Because it is the interactive capacity and nature of a polypeptide that defines that polypeptide's biological functional activity, certain amino acid sequence substitutions can be made in a polypeptide sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a polypeptide with like properties.

In making such changes, the hydropathic index of amino acids can be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a polypeptide is generally understood in the art (Kyte & Doolittle, *J. Mol. Biol.*, 157:105-132, 1982). It is known that certain amino acids can be substituted for other amino acids having a similar hydropathic index or score and still result in a polypeptide with similar biological activity. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. Those indices are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is believed that the relative hydropathic character of the amino acid determines the secondary structure of the resultant polypeptide, which in turn defines the interaction of the polypeptide with other molecules, such as enzymes, substrates, receptors, antibodies, antigens, and the like. It is known in the art that an amino acid can be substituted by another amino acid having a similar hydropathic index and still obtain a functionally equivalent polypeptide. In such changes, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

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Substitution of like amino acids can also be made on the basis of hydrophilicity, particularly where the biological functional equivalent polypeptide or peptide thereby created is intended for use in immunological embodiments. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a polypeptide, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e. with a biological property of the polypeptide.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); proline (-0.5 ± 1); threonine (-0.4); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent polypeptide. In such changes, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine (See Table 1, below). The present invention thus contemplates functional

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or biological equivalents of a polypeptide other than BCL-2 that promotes or inhibits programmed vertebrate cell death as set forth above.

TABLE 1

<u>Original Residue</u>	<u>Exemplary Substitutions</u>
Ala	Gly; Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg
Met	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg
Met	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Biological or functional equivalents of a polypeptide can also be prepared using site-specific mutagenesis. Site-specific mutagenesis is a technique useful in the preparation of second generation polypeptides, or biologically functional equivalent polypeptides or peptides, derived from the sequences thereof, through specific mutagenesis of the underlying DNA. As noted above, such changes can be desirable where amino acid substitutions are desirable. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or

more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by *Adelman, et al. (1983)*. As will be appreciated, the technique typically employs a phage vector which can exist in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (*Messing, et al. 1981*). These phage are commercially available and their use is generally known to those of skill in the art.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector which includes within its sequence a DNA sequence which encodes all or a portion of the polypeptide sequence selected. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of *Crea, et al. (1978)*. This primer is then annealed to the single-stranded vector, and extended by the use of enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells such as *E. coli* cells and clones are selected which include recombinant vectors

bearing the mutation. Commercially available kits come with all the reagents necessary, except the oligonucleotide primers.

A polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death of the invention is not limited to a particular source. As disclosed herein, the techniques and compositions of the present invention provide, for example, the identification and isolation of such peptides from animals as diverse as human and chicken. Thus, the invention provides for the general detection and isolation of the genus of polypeptides from a variety of sources while identifying specifically three species of that genus. It is believed that a number of species of the same family of polypeptides are amenable to detection and isolation using the compositions and methods of the present inventions.

A polypeptide of the present invention is prepared by standard techniques well known to those skilled in the art. Such techniques include, but are not limited to, isolation and purification from tissues known to contain that polypeptide, and expression from cloned DNA that encodes such a polypeptide using transformed cells.

Polypeptides that affect or alter programmed vertebrate cell death or apoptosis are found in virtually all mammals including human. Although it is likely that there exist variations between the structure and function of such polypeptides in different species, where such a difference exists, identification of those differences is well within the skill of an artisan in light of the present invention. Thus, the present invention contemplates a polypeptide other than BCL-2 that promotes or inhibits programmed vertebrate cell death from any vertebrate. A preferred mammal is a human. A preferred vertebrate is a mammal.

III. Expression Vectors

In an alternate embodiment, the present invention provide an expression vector comprising a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death. Preferably, an expression vector of the present invention comprises a polynucleotide that encodes polypeptides BCL-X_L (SEQ ID NOS: 6), BCL-X_S (SEQ ID NO:8) or BCL-X_I (position 510 to 698 of SEQ ID NO:6). In a preferred embodiment, an expression vector of the present invention comprises a polynucleotide that encodes a polypeptide comprising the amino acid residue sequence of SEQ ID NOS: 2, 4, 67, 10 and 9). More preferably, an expression vector of the present invention comprises a polynucleotide comprising the nucleotide base sequence of SEQ ID NOS:1, 3, 6, and 8. In a preferred embodiment, an expression vector of the invention comprises a polynucleotide operatively linked to an enhancer-promoter. More preferably still, an expression vector of the 15 invention comprises a polynucleotide operatively linked to a prokaryotic promoter. Alternatively, an expression vector of the present invention comprises a polynucleotide operatively linked to an enhancer-promoter that is a eukaryotic promoter and further comprises a polyadenylation signal that is positioned 3' of the carboxy-terminal amino acid and within 20 a transcriptional unit of the encoded polypeptide.

A promoter is a region of a DNA molecule typically within about 100 nucleotide pairs in front of (upstream of) the point at which transcription begins (i.e., a transcription start site). That region typically contains several types of DNA sequence elements that are located in 25 similar relative positions in different genes. As used herein, the term "promoter" includes what is referred to in the art as an upstream promoter region, a promoter region or a promoter of a generalized eukaryotic RNA Polymerase II transcription unit.

Another type of discrete transcription regulatory sequence element 30 is an enhancer. An enhancer provides specificity of time, location and

expression level for a particular encoding region (e.g., gene). A major function of an enhancer is to increase the level of transcription of a coding sequence in a cell that contains one or more transcription factors that bind to that enhancer. Unlike a promoter, an enhancer can function when located at variable distances from transcription start sites so long as a promoter is present.

As used herein, the phrase "enhancer-promoter" means a composite unit that contains both enhancer and promoter elements. An enhancer-promoter is operatively linked to a coding sequence that encodes at least one gene product. As used herein, the phrase "operatively linked" means that an enhancer-promoter is connected to a coding sequence in such a way that the transcription of that coding sequence is controlled and regulated by that enhancer-promoter. Means for operatively linking an enhancer-promoter to a coding sequence are well known in the art. As is also well known in the art, the precise orientation and location relative to a coding sequence whose transcription is controlled, is dependent *inter alia* upon the specific nature of the enhancer-promoter. Thus, a TATA box minimal promoter is typically located from about 25 to about 30 base pairs upstream of a transcription initiation site and an upstream promoter element is typically located from about 100 to about 200 base pairs upstream of a transcription initiation site. In contrast, an enhancer can be located downstream from the initiation site and can be at a considerable distance from that site.

An enhancer-promoter used in a vector construct of the present invention can be any enhancer-promoter that drives expression in a cell to be transfected. By employing an enhancer-promoter with well-known properties, the level and pattern of gene product expression can be optimized.

A coding sequence of an expression vector is operatively linked to a transcription terminating region. RNA polymerase transcribes an encoding DNA sequence through a site where polyadenylation occurs.

5 Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions.

Those regions are required for efficient polyadenylation of transcribed messenger RNA (RNA). Transcription-terminating regions are well known in the art. A preferred transcription-terminating region used in an

10 adenovirus vector construct of the present invention comprises a polyadenylation signal of SV40 or the protamine gene.

An expression vector comprises a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death. Such a polypeptide is meant to

15 include a sequence of nucleotide bases encoding a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death sufficient in length to distinguish said segment from a polynucleotide segment encoding a polypeptide that does not affect programmed vertebrate cell death. A polypeptide of the invention can

20 also encode biologically functional polypeptides or peptides which have variant amino acid sequences, such as with changes selected based on considerations such as the relative hydropathic score of the amino acids being exchanged. These variant sequences are those isolated from natural sources or induced in the sequences disclosed herein using a

25 mutagenic procedure such as site-directed mutagenesis.

Preferably, an expression vector of the present invention comprises a polynucleotide that encodes a polypeptide comprising BCL-X_L (SEQ ID NO:6), BCL-X_S (SEQ ID NO:8), BCL-X_I (position 510 to 698 of SEQ ID NO:6) or the amino acid residue sequence of SEQ ID NOS: 2, 4, 7 and 9.

30 An expression vector can include a polypeptide other than BCL-2 SEQ ID

NO:5) that promotes or inhibits programmed vertebrate cell death coding region itself or it can contain coding regions bearing selected alterations or modifications in the basic coding region of such a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed
5 vertebrate cell death. Alternatively, such vectors or fragments can code larger polypeptides or polypeptides which nevertheless include the basic coding region. In any event, it should be appreciated that due to codon redundancy as well as biological functional equivalence, this aspect of the invention is not limited to the particular DNA molecules corresponding to
10 the polypeptide sequences noted above.

Exemplary vectors include the mammalian expression vectors of the pCMV family including pCMV6b and pCMV6c (Chiron Corp., Emeryville CA). In certain cases, and specifically in the case of these individual mammalian expression vectors, the resulting constructs can require co-transfection with a vector containing a selectable marker such as
15 pSV2neo. Via co-transfection into a dihydrofolate reductase-deficient Chinese hamster ovary cell line, such as DG44, clones expressing polypeptides by virtue of DNA incorporated into such expression vectors can be detected.

20 A DNA molecule of the present invention can be incorporated into a vector, a number of techniques which are well known in the art. For instance, bcl-x (SEQ ID NOS:1 and 3), bcl-x_L (SEQ ID NO:6) and bcl-x_S (SEQ ID NO:8) were incorporated into pSFFV-Neo and pBluescript-Sk+ using standard techniques (See Examples herinafter).

25 An expression vector of the present invention is useful both as a means for preparing quantities of the encoding DNA itself, and as a means for preparing the encoded polypeptide. It is contemplated that where a polypeptide of the invention is made by recombinant means, one can employ either prokaryotic or eukaryotic expression vectors as shuttle

systems. However, in that prokaryotic systems are usually incapable of correctly processing precursor polypeptides and, in particular, such systems are incapable of correctly processing membrane associated eukaryotic polypeptides, and since eukaryotic polypeptides are anticipated using the teaching of the disclosed invention, one likely expresses such sequences in eukaryotic hosts. However, even where the DNA segment encodes a eukaryotic polypeptide, it is contemplated that prokaryotic expression can have some additional applicability. Therefore, the invention can be used in combination with vectors which can shuttle between the eukaryotic and prokaryotic cells. Such a system is described herein which allows the use of bacterial host cells as well as eukaryotic host cells.

Where expression of recombinant polypeptide of the present invention is desired and a eukaryotic host is contemplated, it is most desirable to employ a vector such as a plasmid, that incorporates a eukaryotic origin of replication. Additionally, for the purposes of expression in eukaryotic systems, one desires to position the polypeptide encoding sequence adjacent to and under the control of an effective eukaryotic promoter such as promoters used in combination with Chinese hamster ovary cells. To bring a coding sequence under control of a promoter, whether it is eukaryotic or prokaryotic, what is generally needed is to position the 5' end of the translation initiation side of the proper translational reading frame of the polypeptide between about 1 and about 50 nucleotides 3' of or downstream with respect to the promoter chosen. Furthermore, where eukaryotic expression is anticipated, one would typically desire to incorporate an appropriate polyadenylation site into the transcriptional unit which includes the desired polypeptide.

The pCMV plasmids are a series of mammalian expression vectors of particular utility in the present invention. The vectors are designed for use in essentially all cultured cells and work extremely well in SV40-

transformed simian COS cell lines. The pCMV1, 2, 3, and 5 vectors differ from each other in certain unique restriction sites in the polylinker region of each plasmid. The pCMV4 vector differs from these 4 plasmids in containing a translation enhancer in the sequence prior to the polylinker.

5 While they are not directly derived from the pCMV1-5 series of vectors, the functionally similar pCMV6b and c vectors are available from the Chiron Corp. of Emeryville, CA and are identical except for the orientation of the polylinker region which is reversed in one relative to the other.

The universal components of the pCMV plasmids are as follows.

10 The vector backbone is pTZ18R (Pharmacia), and contains a bacteriophage f1 origin of replication for production of single stranded DNA and an ampicillin-resistance gene. The CMV region consists of nucleotides -760 to +3 of the powerful promoter-regulatory region of the human cytomegalovirus (Towne stain) major immediate early gene

15 (*Thomsen et al., 1984; Boshart et al., 1985*). The human growth hormone fragment (hGH) contains transcription termination and polyadenylation signals representing sequences 1533 to 2157 of this gene (*Seenburg, 1982*). There is an *Alu* middle repetitive DNA sequence in this fragment. Finally, the SV40 origin of replication and early region

20 promoter-enhancer derived from the pcD-X plasmid (*HindII to PstI* fragment) described in (*Okayama et al., 1983*). The promoter in this fragment is oriented such that transcription proceeds away from the CMV/hGH expression cassette.

The pCMV plasmids are distinguishable from each other by

25 differences in the polylinker region and by the presence or absence of the translation enhancer. The starting pCMV1 plasmid has been progressively modified to render an increasing number of unique restriction sites in the polylinker region. To create pCMV2, one of two *EcoRI* sites in pCMV1 were destroyed. To create pCMV3, pCMV1 was modified by deleting a

30 short segment from the SV40 region (*StuI to EcoRI*), and in so doing

made unique the *PstI*, *SaI*, and *BamHI* sites in the polylinker. To create pCMV4, a synthetic fragment of DNA corresponding to the 5'-untranslated region of a mRNA transcribed from the CMV promoter was added C. The sequence acts as a translational enhancer by decreasing the requirements for initiation factors in polypeptide synthesis (*Jobling et al.*, 1987); *Browning et al.*, 1988). To create pCMV5, a segment of DNA (*HpaI* to *EcoRI*) was deleted from the SV40 origin region of pCMV1 to render unique all sites in the starting polylinker.

The pCMV vectors have been successfully expressed in simian COS cells, mouse L cells, CHO cells, and HeLa cells. In several side by side comparisons they have yielded 5- to 10-fold higher expression levels in COS cells than SV40-based vectors. The pCMV vectors have been used to express the LDL receptor, nuclear factor 1, Gs alpha polypeptide, polypeptide phosphatase, synaptophysin, synapsin, insulin receptor, influenza hemmagglutinin, androgen receptor, sterol 26-hydroxylase, steroid 17- and 21-hydroxylase, cytochrome P-450 oxidoreductase, beta-adrenergic receptor, folate receptor, cholesterol side chain cleavage enzyme, and a host of other cDNAs. It should be noted that the SV40 promoter in these plasmids can be used to express other genes such as dominant selectable markers. Finally, there is an ATG sequence in the polylinker between the *HindIII* and *PstI* sites in pCMU that can cause spurious translation initiation. This codon should be avoided if possible in expression plasmids. A paper describing the construction and use of the parenteral pCMV1 and pCMV4 vectors has been published (Anderson et al., 1989b).

IV. Transfected Cells.

In yet another embodiment, the present invention provides recombinant host cells transformed or transfected with a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that inhibits or promotes programmed vertebrate cell death, as well as transgenic cells

derived from those transformed or transfected cells. Preferably, a recombinant host cell of the present invention is transfected with a polynucleotide containing sequences of SEQ ID NOS: 1, 3, 6, or 8.

Means of transforming or transfecting cells with exogenous

5 polynucleotide such as DNA molecules are well known in the art and include techniques such as calcium-phosphate- or DEAE-dextran-mediated transfection, protoplast fusion, electroporation, liposome mediated transfection, direct microinjection and adenovirus infection (*Sambrook, Fritsch and Maniatis, 1989*).

10 The most widely used method is transfection mediated by either calcium phosphate or DEAE-dextran. Although the mechanism remains obscure, it is believed that the transfected DNA enters the cytoplasm of the cell by endocytosis and is transported to the nucleus. Depending on the cell type, up to 90% of a population of cultured cells can be

15 transfected at any one time. Because of its high efficiency, transfection mediated by calcium phosphate or DEAE-dextran is the method of choice for studies that require transient expression of the foreign DNA in large numbers of cells. Calcium phosphate-mediated transfection is also used to establish cell lines that integrate copies of the foreign DNA, which are

20 usually arranged in head-to-tail tandem arrays into the host cell genome.

In the protoplast fusion method, protoplasts derived from bacteria carrying high numbers of copies of a plasmid of interest are mixed directly with cultured mammalian cells. After fusion of the cell membranes (usually with polyethylene glycol), the contents of the bacteria are delivered into the cytoplasm of the mammalian cells and the plasmid DNA is transported to the nucleus. Protoplast fusion is not as efficient as transfection for many of the cell lines that are commonly used for transient expression assays, but it is useful for cell lines in which endocytosis of DNA occurs inefficiently. Protoplast fusion frequently

yields multiple copies of the plasmid DNA tandemly integrated into the host chromosome.

The application of brief, high-voltage electric pulses to a variety of mammalian and plant cells leads to the formation of nanometer-sized pores in the plasma membrane. DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of the redistribution of membrane components that accompanies closure of the pores. Electroporation can be extremely efficient and can be used both for transient expression of cloned genes and for establishment of cell lines that carry integrated copies of the gene of interest. Electroporation, in contrast to calcium phosphate-mediated transfection and protoplast fusion, frequently gives rise to cell lines that carry one, or at most a few, integrated copies of the foreign DNA.

Liposome transfection involves encapsulation of DNA and RNA within liposomes, followed by fusion of the liposomes with the cell membrane. The mechanism of how DNA is delivered into the cell is unclear but transfection efficiencies can be as high as 90%.

Direct microinjection of a DNA molecule into nuclei has the advantage of not exposing DNA to cellular compartments such as low-pH endosomes. Microinjection is therefore used primarily as a method to establish lines of cells that carry integrated copies of the DNA of interest.

The use of adenovirus as a vector for cell transfection is well known in the art. Adenovirus vector-mediated cell transfection has been reported for various cells (*Stratford-Perricaudet, et al. 1992*).

A transfected cell can be prokaryotic or eukaryotic. Preferably, the host cells of the invention are eukaryotic host cells. A preferred recombinant host cell of the invention is a murine FL5.12 cell. Where it is

of interest to produce a human polypeptide other than BCL-2 that promotes or inhibits programmed vertebrate cell deaths, cultured mammalian or human cells are of particular interest.

In another aspect, a recombinant host cell of the present invention 5 is a prokaryotic host cell. Preferably, a recombinant host cell is a bacterial cell of a strain of *Escherichia coli*. In general, prokaryotes are preferred for the initial cloning of DNA sequences and constructing the vectors useful in the invention. For example, *E. coli* K12 strains can be particularly useful. Other microbial strains which can be used include 10 *E. coli* B, and *E. coli* X1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes can also be used for expression. The aforementioned strains, as well as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325), bacilli such as *Bacillus subtilis*, or other enterobacteriaceae 15 such as *Salmonella typhimurium* or *Serratia marcescens*, and various *Pseudomonas* species can be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell 20 are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* can be transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, et al. 1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying 25 transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own polypeptides.

Those promoters most commonly used in recombinant DNA construction include the β -lactamase (penicillinase) and lactose promoter systems (*Chang, et al. 1978; Itakura, et al. 1977; Goeddel, et al. 1979; Goeddel, et al. 1980*) and a tryptophan (TRP) promoter system (EPO Appl. 5 Publ. No. 0036776; *Siebwenlist et al., 1980*). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to introduce functional promoters into plasmid vectors (*Siebwenlist, et al. 1980*).

10 In addition to prokaryotes, eukaryotic microbes, such as yeast can also be used. *Saccharomyces cerevisiae* or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in 15 *Saccharomyces*, the plasmid YRp7, for example, is commonly used (*Stinchcomb, et al. 1979; Kingsman, et al. 1979; Tschemper, et al. 1980*). This plasmid already contains the *trpl* gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (*Jones, 1977*). The presence of the *trpl* lesion as a characteristic of the yeast host cell 20 genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoter sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (*Hitzeman, et al. 1980*) or other glycolytic enzymes (*Hess, et al. 1968; Holland, et al. 1978*) such as enolase, 25 glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these 30 genes are also introduced into the expression vector downstream from

the sequences to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase,
5 degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin or replication and termination sequences is suitable.

10 In addition to microorganisms, cultures of cells derived from multicellular organisms can also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine
15 procedure in recent years (*Kruse and Peterson, 1973*). Examples of such useful host cell lines are AtT-20, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COSM6, COS-7, 293 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located upstream of the
20 gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often derived from viral material. For example, commonly
25 used promoters are derived from polyoma, Adenovirus 2, Cytomegalovirus and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (*Fiers, et al. 1978*). Smaller or larger
30 SV40 fragments can also be used, provided there is included the

approximately 250 bp sequence extending from the *Hind*III site toward the *Bgl*I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such
5 control sequences are compatible with the host cell systems.

An origin of replication can be provided with by construction of the vector to include an exogenous origin, such as can be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV, CMV) source, or can be provided by the host cell chromosomal replication mechanism. If the
10 vector is integrated into the host cell chromosome, the latter is often sufficient.

V. Preparing Recombinant Polypeptides Other Than BCL-2 (SEQ IDNO:5) That Affect Programmed vertebrate cell death.

In yet another embodiment, the present invention contemplates a
15 process of preparing a polypeptide other than BCL-2 (SEQ IDNO:5) that affects programmed vertebrate cell death comprising transfecting cells with a polynucleotide that encodes that polypeptide to produce a transformed host cell; and maintaining the transformed host cell under biological conditions sufficient for expression of the polypeptide.
20 Preferably, the transformed host cells is a eukaryotic cell. Alternatively, the host cells is a prokaryotic cell. Preferred prokaryotic cells are bacterial cells of the DH5 α strain of *Escherichia coli*. Even more preferably, the polynucleotide transfected into the transformed cells comprises the nucleotide base sequence of SEQ ID NOS 1 and 3
25 (Figure 1). Most preferably, transfection is accomplished using a hereinbefore disclosed expression vector.

A host cell used in the process is capable of expressing a functional, polypeptide of the present invention. A preferred host cell is a Chinese hamster ovary cell. However, a variety of cells are amenable to a

process of the invention, for instance, yeast cells, human cell lines, and other eukaryotic cell lines known well to those of the art.

Following transfection, the cell is maintained under culture conditions for a period of time sufficient for expression of a polypeptide other than BCL-2 (SEQ IDNO:5) that promotes or inhibits programmed vertebrate cell death. Culture conditions are well known in the art and include ionic composition and concentration, temperature, pH and the like. Typically, transfected cells are maintained under culture conditions in a culture medium. Suitable medium for various cell types are well known in the art. In a preferred embodiment, temperature is from about 20°C to about 50°C, more preferably from about 30°C to about 40°C and, even more preferably about 37°C.

pH is preferably from about a value of 6.0 to a value of about 8.0, more preferably from about a value of about 6.8 to a value of about 7.8 and, most preferably about 7.4. Osmolality is preferably from about 200 milliosmols per liter (mosm/L) to about 400 mosm/l and, more preferably from about 290 mosm/L to about 310 mosm/L. Other biological conditions needed for transfection and expression of an encoded protein are well known in the art.

Transfected cells are maintained for a period of time sufficient for expression of a polypeptide other than BCL-2 that promotes or inhibits programmed vertebrate cell death. A suitable time depends *inter alia* upon the cell type used and is readily determinable by a skilled artisan. Typically, maintenance time is from about 2 to about 14 days.

Recombinant polypeptide is recovered or collected either from the transfected cells or the medium in which those cells are cultured. Recovery comprises isolating and purifying the polypeptide. Isolation and purification techniques for polypeptides are well known in the art and

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include such procedures as precipitation, filtration, chromatography, electrophoresis and the like.

VI. Antibodies.

In still another embodiment, the present invention provides an 5 antibody immunoreactive with a polypeptide of the present invention. Preferably, an antibody of the invention is a monoclonal antibody. Means for preparing and characterizing antibodies are well known in the art (See, e.g., *Antibodies "A Laboratory Manual*, E. Howell and D. Lane, Cold Spring Harbor Laboratory, 1988).

10 Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide or polynucleotide of the present invention, and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a rabbit, a 15 mouse, a rat, a hamster or a guinea pig. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given polypeptide or polynucleotide 20 may vary in its immunogenicity. It is often necessary therefore to couple the immunogen (e.g., a polypeptide or polynucleotide) of the present invention with a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers.

25 Means for conjugating a polypeptide or a polynucleotide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, immunogenicity to a particular immunogen can be enhanced by the use of non-specific stimulators of the immune response known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant, incomplete Freund's
5 adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen used of the production of polyclonal antibodies varies *inter alia*, upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal,
10 intravenous and intraperitoneal. The production of polyclonal antibodies is monitored by sampling blood of the immunized animal at various points following immunization. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored.

15 In another aspect, the present invention contemplates a process of producing an antibody immunoreactive with a polypeptide other than BCL-2 (SEQ IDNO:5) that promotes or inhibits programmed vertebrate cell death comprising the steps of (a) transfecting recombinant host cells with a polynucleotide that encodes that polypeptide; (b) culturing the host cells
20 under conditions sufficient for expression of the polypeptide; (c) recovering the polypeptide; and (d) preparing antibodies to the polypeptide. Even more preferably, the present invention provides antibodies prepared according to the process described above.

A monoclonal antibody of the present invention can be readily prepared through use of well-known techniques such as those exemplified
25 in U.S. Pat. No 4,196,265, herein incorporated by reference. Typically, a technique involves first immunizing a suitable animal with a selected antigen (e.g., a polypeptide or polynucleotide of the present invention) in a manner sufficient to provide an immune response. Rodents such as

mice and rats are preferred animals. Spleen cells from the immunized animal are then fused with cells of an immortal myeloma cell. Where the immunized animal is a mouse, a preferred myeloma cell is a murine NS-1 myeloma cell.

5 The fused spleen/myeloma cells are cultured in a selective medium to select fused spleen/myeloma cells from the parental cells. Fused cells are separated from the mixture of non-fused parental cells, for example, by the addition of agents that block the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are

10 aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides. Where azaserine is used, the media

15 is supplemented with hypoxanthine.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants for reactivity with an antigen-polypeptides. The selected clones can then be propagated indefinitely to provide the monoclonal antibody.

20 By way of specific example, to produce an antibody of the present invention, mice are injected intraperitoneally with between about 1-200 µg of an antigen comprising a polypeptide of the present invention.

25 B lymphocyte cells are stimulated to grow by injecting the antigen in association with an adjuvant such as complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*). At some time (e.g., at least two weeks)

after the first injection, mice are boosted by injection with a second dose of the antigen mixed with incomplete Freund's adjuvant.

A few weeks after the second injection, mice are tail bled and the sera titered by immunoprecipitation against radiolabeled antigen.

5 Preferably, the process of boosting and titering is repeated until a suitable titer is achieved. The spleen of the mouse with the highest titer is removed and the spleen lymphocytes are obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

10 Mutant lymphocyte cells known as myeloma cells are obtained from laboratory animals in which such cells have been induced to grow by a variety of well-known methods. Myeloma cells lack the salvage pathway of nucleotide biosynthesis. Because myeloma cells are tumor cells, they can be propagated indefinitely in tissue culture, and are thus 15 denominated immortal. Numerous cultured cell lines of myeloma cells from mice and rats, such as murine NS-1 myeloma cells, have been established.

20 Myeloma cells are combined under conditions appropriate to foster fusion with the normal antibody-producing cells from the spleen of the mouse or rat injected with the antigen/polypeptide of the present invention. Fusion conditions include, for example, the presence of polyethylene glycol. The resulting fused cells are *hybridoma* cells. Like myeloma cells, hybridoma cells grow indefinitely in culture.

25 Hybridoma cells are separated from unfused myeloma cells by culturing in a selection medium such as HAT media (hypoxanthine, aminopterin, thymidine). Unfused myeloma cells lack the enzymes necessary to synthesize nucleotides from the salvage pathway because they are killed in the presence of aminopterin, methotrexate, or azaserine.

Unfused lymphocytes also do not continue to grow in tissue culture. Thus, only cells that have successfully fused (hybridoma cells) can grow in the selection media.

Each of the surviving hybridoma cells produces a single antibody.

5 These cells are then screened for the production of the specific antibody immunoreactive with an antigen/polypeptide of the present invention. Single cell hybridomas are isolated by limiting dilutions of the hybridomas. The hybridomas are serially diluted many times and, after the dilutions are allowed to grow, the supernatant is tested for the presence of the 10 monoclonal antibody. The clones producing that antibody are then cultured in large amounts to produce an antibody of the present invention in convenient quantity.

By use of a monoclonal antibody of the present invention, specific polypeptides and polynucleotide of the invention can be recognized as 15 antigens, and thus identified. Once identified, those polypeptides and polynucleotide can be isolated and purified by techniques such as *antibody-affinity chromatography*. In antibody-affinity chromatography, a monoclonal antibody is bound to a solid substrate and exposed to a solution containing the desired antigen. The antigen is removed from the 20 solution through an immunospecific reaction with the bound antibody. The polypeptide or polynucleotide is then easily removed from the substrate and purified.

VII. Pharmaceutical Compositions.

In a preferred embodiment, the present invention provides 25 pharmaceutical compositions comprising a polypeptide or polynucleotide of the present invention and a physiologically acceptable carrier. More preferably, a pharmaceutical composition comprises polypeptide BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4) or a polynucleotide that encodes those polypeptides.

A composition of the present invention is typically administered parenterally in dosage unit formulations containing standard, well-known nontoxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes intravenous, intra-muscular, intraarterial injection, or infusion techniques.

5 Injectable preparations, for example sterile injectable aqueous or oleaginous suspensions, are formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, 10 as a solution in 1,3-butanediol.

15 Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

20 Preferred carriers include neutral saline solutions buffered with phosphate, lactate, Tris, and the like. Of course, one purifies the vector sufficiently to render it essentially free of undesirable contaminants, such as defective interfering adenovirus particles or endotoxins and other pyrogens such that it does not cause any untoward reactions in the individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium 25 chloride gradient centrifugation.

A carrier can also be a liposome. Means for using liposomes as delivery vehicles are well known in the art [See, e.g. *Gabizon et al., 1990; Ferruti et al., 1986; and Ranade, V.V., 1989*].

A transfected cell can also serve as a carrier. By way of example, a liver cell can be removed from an organism, transfected with a polynucleotide of the present invention using methods set forth above and then the transfected cell returned to the organism (e.g. injected 5 intravascularly).

VIII. Detecting a Polynucleotide or a Polypeptide of the Present Invention.

Alternatively, the present invention provides a process of detecting a polypeptide of the present invention, wherein the process comprises 10 immunoreacting the polypeptides with antibodies prepared according to the process described above to form antibody-polypeptide conjugates, and detecting the conjugates.

In yet another embodiment, the present invention contemplates a process of detecting messenger RNA transcripts that encode a 15 polypeptide of the present invention, wherein the process comprises (a) hybridizing the messenger RNA transcripts with polynucleotide sequences that encode the polypeptide to form duplexes; and (b) detecting the duplex. Alternatively, the present invention provides a process of detecting DNA molecules that encode a polypeptide of the present invention, wherein the process comprises (a) hybridizing DNA molecules 20 with a polynucleotide that encodes that polypeptide to form duplexes; and (b) detecting the duplexes.

IX. Screening Assays

In yet another aspect, the present invention contemplates a 25 process of screening substances for their ability to affect programmed vertebrate cell death comprising the steps of providing a cell that contains a functional polypeptide of the present invention and testing the ability of selected substances to affect programmed vertebrate cell death of that cell.

Utilizing the methods and compositions of the present invention, screening assays for the testing of candidate substances can be derived. A candidate substance is a substance which potentially can promote or inhibit programmed vertebrate cell death, by binding or other 5 intramolecular interaction, with a polypeptide other than BCL-2 that promotes or inhibits programmed vertebrate cell death.

A screening assay of the present invention generally involves determining the ability of a candidate substance to affect the viability of a target cell (susceptibility to programmed vertebrate cell death), such as 10 the screening of candidate substances to identify those that inhibit or promote programmed vertebrate cell death. Target cells can be either naturally occurring cells known to contain a polypeptide of the present invention or transformed cell produced in accordance with a process of transformation set forth hereinbefore.

15 As is well known in the art, a screening assay provides a cell under conditions suitable for testing programmed vertebrate cell death. These conditions include but are not limited to pH, temperature, tonicity, the presence of relevant factors involved in programmed vertebrate cell death (e.g., growth factor, IL-3), and relevant modifications to the polypeptide 20 such as glycosylation or prenylation. It is contemplated that a polypeptide of the present invention can be expressed and utilized in a prokaryotic or eukaryotic cell. The host cell can also be fractionated into sub-cellular fractions where the receptor can be found. For example, cells expressing the polypeptide can be fractionated into the nuclei, the 25 endoplasmic reticulum, vesicles, or the membrane surfaces of the cell.

pH is preferably from about a value of 6.0 to a value of about 8.0, more preferably from about a value of about 6.8 to a value of about 7.8 and, most preferably about 7.4. In a preferred embodiment, temperature is from about 20°C to about 50°C, more preferably from about 30°C to

about 40°C and, even more preferably about 37°C. Osmolality is preferably from about 5 milliosmols per liter (mosm/L) to about 400 mosm/l and, more preferably from about 200 milliosmols per liter to about 400 mosm/l and, even more preferably from about 290 mosm/L to about 5 310 mosm/L. The presence of factors can be required for the proper testing of programmed vertebrate cell death in specific cells. Such factors include, for example, the presence and absence (withdrawal) of growth factor, interleukins, or colony stimulating factors.

10 In one embodiment, a screening assay is designed to be capable of discriminating candidate substances having selective ability to interact with one or more of the polypeptides of the present invention but which polypeptides are without a substantially overlapping activity with another of those polypeptides identified herein.

A. Screening assays for a polypeptide of the present invention.

15 The present invention provides a process of screening a biological sample for the presence of a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death. A biological sample to be screened can be a biological fluid such as extracellular or intracellular fluid or a cell or tissue extract or homogenate. 20 A biological sample can also be an isolated cell (e.g., in culture) or a collection of cells such as in a tissue sample or histology sample. A tissue sample can be suspended in a liquid medium or fixed onto a solid support such as a microscope slide.

25 In accordance with a screening assay process, a biological sample is exposed to an antibody immunoreactive with the polypeptide whose presence is being assayed. Typically, exposure is accomplished by forming an admixture in a liquid medium that contains both the antibody and the candidate polypeptide. Either the antibody or the sample with the

polypeptide can be affixed to a solid support (e.g., a column or a microtiter plate).

The biological sample is exposed to the antibody under biological reaction conditions and for a period of time sufficient for antibody-polypeptide conjugate formation. Biological reaction conditions include 5 ionic composition and concentration, temperature, pH and the like.

Ionic composition and concentration can range from that of distilled water to a 2 molal solution of NaCl. Preferably, osmolality is from about 100 mosmols/l to about 400 mosmols/l and, more preferably from about 10 200 mosmols/l to about 300 mosmols/l. Temperature preferably is from about 4°C to about 100°C, more preferably from about 15°C to about 50°C and, even more preferably from about 25°C to about 40°C. pH is preferably from about a value of 4.0 to a value of about 9.0, more 15 preferably from about a value of 6.5 to a value of about 8.5 and, even more preferably from about a value of 7.0 to a value of about 7.5. The only limit on biological reaction conditions is that the conditions selected allow for antibody-polypeptide conjugate formation and that the conditions do not adversely affect either the antibody or the polypeptide.

Exposure time will vary *inter alia* with the biological conditions 20 used, the concentration of antibody and polypeptide and the nature of the sample (e.g., fluid or tissue sample). Means for determining exposure time are well known to one of ordinary skill in the art. Typically, where the sample is fluid and the concentration of polypeptide in that sample is about 10^{-10} M, exposure time is from about 10 minutes to about 200 25 minutes.

The presence of polypeptide in the sample is detected by detecting the formation and presence of antibody-polypeptide conjugates. Means for detecting such antibody-antigen (e.g., receptor polypeptide)

conjugates or complexes are well known in the art and include such procedures as centrifugation, affinity chromatography and the like, binding of a secondary antibody to the antibody-candidate receptor complex.

5 In one embodiment, detection is accomplished by detecting an indicator affixed to the antibody. Exemplary and well known such indicators include radioactive labels (e.g., ^{32}P , ^{125}I , ^{14}C), a second antibody or an enzyme such as horse radish peroxidase. Means for affixing indicators to antibodies are well known in the art. Commercial kits are
10 available.

B. Screening assay for anti-polypeptide antibody.

In another aspect, the present invention provides a process of screening a biological sample for the presence of antibodies immunoreactive with a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death (e.g., BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4)). In accordance with such a process, a biological sample is exposed to a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death under biological conditions and for a period of time sufficient for antibody-polypeptide conjugate formation and the formed conjugates are detected.
15
20

C. Screening assay for polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death.

25 A DNA molecule and, particularly a probe molecule, can be used for hybridizing as an oligonucleotide probe to a DNA source suspected of encoding a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death. The probing is usually accomplished by hybridizing the oligonucleotide to a DNA source
30 suspected of possessing an apoptosis gene. In some cases, the probes

constitute only a single probe, and in others, the probes constitute a collection of probes based on a certain amino acid sequence or sequences of the polypeptide and account in their diversity for the redundancy inherent in the genetic code.

5 A suitable source of DNA for probing in this manner is capable of expressing a polypeptide of the present invention and can be a genomic library of a cell line of interest. Alternatively, a source of DNA can include total DNA from the cell line of interest. Once the hybridization process of the invention has identified a candidate DNA segment, one
10 confirms that a positive clone has been obtained by further hybridization, restriction enzyme mapping, sequencing and/or expression and testing.

15 Alternatively, such DNA molecules can be used in a number of techniques including their use as: (1) diagnostic tools to detect normal and abnormal DNA sequences in DNA derived from patient's cells; (2) means for detecting and isolating other members of the polypeptide family and related polypeptides from a DNA library potentially containing such sequences; (3) primers for hybridizing to related sequences for the purpose of amplifying those sequences; (4) primers for altering native apoptosis DNA sequences; as well as other techniques which rely on the similarity of the DNA sequences to those of the DNA segments herein
20 disclosed.

25 As set forth above, in certain aspects, DNA sequence information provided by the invention allows for the preparation of relatively short DNA (or RNA) sequences (e.g., probes) that specifically hybridize to encoding sequences of a selected apoptosis gene. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of the encoding sequence for a polypeptide of this invention. The ability of such nucleic acid probes to specifically hybridize to other encoding sequences lend them particular utility in a variety of

embodiments. Most importantly, the probes can be used in a variety of assays for detecting the presence of complementary sequences in a given sample. However, uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for
5 use in preparing other genetic constructions.

To provide certain of the advantages in accordance with the invention, a preferred nucleic acid sequence employed for hybridization studies or assays includes probe sequences that are complementary to at least a 14 to 40 or so long nucleotide stretch of a nucleic acid sequence
10 of the present invention, such as that shown in Figure 1. A size of at least 14 nucleotides in length helps to ensure that the fragment is of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 14 bases in length are generally preferred, though, to
15 increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 14 to 20 nucleotides, or even longer where desired. Such fragments can be readily prepared by, for example, directly
20 synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102, herein incorporated by reference, or by introducing selected sequences into recombinant vectors for recombinant production.

Accordingly, a nucleotide sequence of the present invention can be
25 used for its ability to selectively form duplex molecules with complementary stretches of the gene. Depending on the application envisioned, one employs varying conditions of hybridization to achieve varying degrees of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one typically
30 employs relatively stringent conditions to form the hybrids. For example,

one selects relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M NaCl at temperatures of 50°C to 70°C. Such conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

5 Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate polypeptide coding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions are typically needed to allow formation

10 of the heteroduplex. Under such circumstances, one employs conditions such as 0.15M-0.9M salt, at temperatures ranging from 20°C to 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by

15 the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

20 In certain embodiments, it is advantageous to employ a nucleic acid sequence of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one likely employs an enzyme tag such a urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, calorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or

25

spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein are useful both as reagents in solution hybridization as well as in 5 embodiments employing a solid phase. In embodiments involving a solid phase, the sample containing test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions depend *inter alia* on the 10 particular circumstances based on the particular criteria required (depending, for example, on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantified, by 15 means of the label.

X. Assay kits.

In another aspect, the present invention contemplates diagnostic assay kits for detecting the presence of a polypeptide of the present invention in biological samples, where the kits comprise a first container 20 containing a first antibody capable of immunoreacting with the polypeptide, with the first antibody present in an amount sufficient to perform at least one assay. Preferably, the assay kits of the invention further comprise a second container containing a second antibody that immunoreacts with the first antibody. More preferably, the antibodies 25 used in the assay kits of the present invention are monoclonal antibodies. Even more preferably, the first antibody is affixed to a solid support. More preferably still, the first and second antibodies comprise an indicator, and, preferably, the indicator is a radioactive label or an enzyme.

The present invention also contemplates a diagnostic kit for screening agents. Such a kit can contain a polypeptide of the present invention. The kit can contain reagents for detecting an interaction between an agent and a receptor of the present invention. The provided reagent can be radiolabelled. The kit can contain a known radiolabelled agent capable of binding or interacting with a receptor of the present invention.

In an alternative aspect, the present invention provides diagnostic assay kits for detecting the presence, in biological samples, of a polynucleotide that encodes a polypeptide of the present invention, the kits comprising a first container that contains a second polynucleotide identical or complementary to a segment of at least 10 contiguous nucleotide bases of SEQ ID NOS: 1 and 3.

In another embodiment, the present invention contemplates diagnostic assay kits for detecting the presence, in a biological sample, of antibodies immunoreactive with a polypeptide of the present invention, the kits comprising a first container containing a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death that immunoreacts with the antibodies, with the polypeptide present in an amount sufficient to perform at least one assay. The reagents of the kit can be provided as a liquid solution, attached to a solid support or as a dried powder. Preferably, when the reagent is provided in a liquid solution, the liquid solution is an aqueous solution. Preferably, when the reagent provided is attached to a solid support, the solid support can be chromatograph media or a microscope slide. When the reagent provided is a dry powder, the powder can be reconstituted by the addition of a suitable solvent. The solvent can be provided.

XI. Treatment of Programmed Cell Death (Apoptosis) with Gene Therapy

In this example, bcl-x_L (SEQ ID NO:6), bcl-x_S (SEQ ID NO:8), or bcl-x₁ (position 510 to 698 of SEQ ID NO:6) gene therapy directed toward
5 the prevention or treatment of apoptosis is described. These cells include but are not limited to neuronal cells, cells of the immune system, and cancerous or tumorous cells.

In yet another aspect, the present invention contemplates a process of altering programmed cell death in a cell comprising the steps
10 of:

- (a) delivering to the cell an effective amount of a DNA molecule comprising a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that inhibits or promotes vertebrate programmed cell death; and
- 15 (b) maintaining the cell under conditions sufficient for expression of said polypeptide.

In a preferred embodiment, the polypeptide is BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X₁ (SEQ ID NO:4). Delivery is preferably accomplished by injecting the DNA molecule into the cell.
20 Where the cell is in a subject delivering is preferably administering the DNA molecule into the circulatory system of the subject. In a preferred embodiment, administering comprises the steps of:

- (a) providing a vehicle that contains the DNA molecule; and
- (b) administering the vehicle to the subject.

25 A vehicle is preferably a cell transformed or transfected with the DNA molecule or a transfected cell derived from such a transformed or transfected cell. An exemplary and preferred transformed or transfected cell is a leukocyte such as a tumor infiltrating lymphocyte or a T cell or a tumor cell from the tumor being treated. Means for transforming or

transfected a cell with a DNA molecule of the present invention are set forth above.

Human lymphocytes can also be transfected with radiation-inducible plasmid constructs using existing technology including retroviral
5 mediated gene transfer (Overell, et al., 1991; Fauser, 1991). In an exemplary embodiment, LAK cells which tend to home in on the tumor site in question with some degree of preference though as is well known, they will also distribute themselves in the body in other locations, may be used to target tumors. Indeed, one of the most important advantages of
10 the radiation inducible system is that only those LAK cells, which are in the radiation field will be activated and will have their exogenously introduced lymphokine genes activated. Thus, for the case of LAK cells, there is no particular need for any further targeting.

Alternatively, the vehicle is a virus or an antibody that specifically
15 infects or immunoreacts with an antigen of the tumor. Retroviruses used to deliver the constructs to the host target tissues generally are viruses in which the 3' LTR (linear transfer region) has been inactivated. That is, these are enhancerless 3'LTR's, often referred to as SIN (self-inactivating viruses) because after productive infection into the host cell, the 3'LTR is transferred to the 5' end and both viral LTR's are inactive with respect to
20 transcriptional activity. A use of these viruses well known to those skilled in the art is to clone genes for which the regulatory elements of the cloned gene are inserted in the space between the two LTR's. An advantage of a viral infection system is that it allows for a very high level
25 of infection into the appropriate recipient cell, e.g., LAK cells.

The viral constructs are delivered into a host by any method that causes the constructs to reach the cells of the target tissue, while preserving the characteristics of the construct used in this invention. By way of example, a rat glioma cell line, C6-BU-1, showed differential

susceptibility to herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), namely, all the HSV-1 strains tested so far persisted in this cell line but the HSV-2 strains did not (Sakihama, et al., 1991). C6-BU-1 cells consist of subpopulations heterogeneous in susceptibility to HSV-1 which may be
5 possibly interchangeable. Furthermore, growth of tumors produced from C6-derived cells bearing the HSV-1 tk gene, but no parental C6 cells, could be inhibited by intraperitoneal administration of ganciclovir (Ezzeddine, et al., 1991). This work demonstrated the effectiveness of the thymidine kinase expressed by the HSV-1 tk gene in sensitizing brain
10 tumor cells to the toxic effects of nucleoside analogs. Retrovirus vectors should thus prove useful in the selective delivery of this killer gene to dividing tumor cells in the nervous system, where most endogenous cells are not dividing. Radiation will be used to enhance the specificity of delivery or activation of transcription of the tk gene only in irradiated
15 areas.

Antibodies have been used to target and deliver DNA molecules. An N-terminal modified poly-L-lysine (NPLL)-antibody conjugate readily forms a complex with plasmid DNA (Trubetskoy et al., 1992). A complex of monoclonal antibodies against a cell surface thrombomodulin
20 conjugated with NPLL was used to target a foreign plasmid DNA to an antigen-expressing mouse lung endothelial cell line and mouse lung. Those targeted endothelial cells expressed the product encoded by that foreign DNA.

Target cells for gene therapy can be normal cells or cells not under
25 the proper control of its constitutive genes. For example, many cells die during normal development and self-maintenance. Cancerous or tumorous tissues develop when cells fail to die. Further, neurodegenerative diseases have been implicated with premature neuronal

cell death. In addition, premature death of immune system cells have been implicated in autoimmune diseases.

A preferred neuronal cell is any cell of the central nervous system. This neuronal cell type can be a normal neuron or a neuron about to undergo apoptosis. In particular, neuronal cells implicated in neurodegenerative diseases (e.g., such as Parkinson's disease, Amyotrophic Lateral Sclerosis, and Multiple Sclerosis) are contemplated.

A preferred immune cell is any cell of the immune system. This cell type can be a normal immune system cell or a cell about to undergo apoptosis. It is contemplated that this cell includes but is not limited to B and T lymphocytes, leucocytes and thymocytes.

A preferred cancerous or tumorous cell is any cancerous or tumorous cell. This cell type can be any cell which does not undergo apoptosis. It is contemplated that cancerous cells include but is not limited to cells from prostate cancer, breast cancer, cancers of the immune system, bone cancers, and tumors of the central nervous system.

An expression vector containing bcl-x_L (SEQ ID NO:6), bcl-x_S (SEQ ID NO:8), or bcl-x_I (position 510 to 698 of SEQ ID NO:6) can be introduced into neuronal cells, cancerous cells, cells of the immune system or other cells in which treatment of apoptosis is desired. One of ordinary skill in the art can choose an appropriate vector for the target cell type.

By way of specific example, mutated HSV-1 virus can be used as a vector for introduction of the gene into neuronal cells. It is also envisioned that this embodiment of the present invention can be practiced using alternative viral or phage vectors, including retroviral vectors and vaccinia viruses whose genome has been manipulated in alternative ways

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so as to render the virus non-pathogenic. Methods for creating such a viral mutation are set forth in detail in U.S. Patent No. 4,769,331, incorporated herein by reference. It is also contemplated that multi-potent neural cell lines can be used to deliver the bcl-x_L or bcl-x_S gene to the CNS. These procedures involve taking cells of fetal or postnatal CNS origin, immortalizing and transforming them in vitro and transplanting the cells back into the mouse brain. These cells, after engraftment, follow the migration pattern and environmental cue of normal brain cell development and differentiate in a nontumorigenic, cytoarchitecturally appropriate manner. This work has been exemplified in several articles notably Snyder et al., Cell, 68: 33-51, 1992 and Ranfranz et al., Cell, 66: 713-729, 1991. Utilizing appropriately modified techniques, it is possible to introduce the bcl-x_L or bcl-x_S gene alone or in combination with other genes of interest into the cells and engraft. Such a procedure allows the delivery of the genes to its natural site. Proper expression of the bcl-x_L, bcl-x_S, or bcl-x₁ gene in these neurons should result in prevention of cell death in neurodegeneration and preserving cells carrying foreign genes suitable for gene therapy.

20 XII. Treatment of Programmed Cell Death (Apoptosis) with a Polypeptide of the Present Invention

As an alternative to the gene therapy methods described for exemplary purposes in Examples 2 and 3, neuronal cells undergoing or about to undergo programmed cell death can also be treated with the protein expressed by the bcl-x_L, bcl-x_S or bcl-x₁ gene, i.e. BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X₁ (SEQ ID NO:4). Alternatively, a biological functional equivalent protein could be used in such treatment.

For example, BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9), or BCL-X₁ (SEQ ID NO:4) is isolated from cells expressing the protein and purified using conventional chromatography purification and immunoaffinity purification methods described by Ackerman et al. (J. Virol. 58: 843-850, 1986, incorporated herein by reference). The purified protein is next

combined with a pharmaceutically appropriate carrier, such as buffered saline or purified distilled water. For administration, the pharmaceutical composition can be injected in one of several ways, as appropriate: (i) intraspinal injection; (ii) intraventricular injection; (iii) direct injection into the area containing the neurons undergoing or about to undergo programmed cell death or any other appropriate method of administration understood by those skilled in the art. Such treatment would be particularly appropriate in the surgical repair of severed peripheral nerves, and the use of proteins as therapeutic agents is well within the current level of skill in the medical arts in light of the present specification.

The following examples have been included to illustrate preferred modes of the invention. Certain aspects of the following examples are described in terms of techniques and procedures found or contemplated by the present inventors to work well in the practice of the invention. These examples are exemplified through the use of standard laboratory practices of the inventor. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following examples are intended to be exemplary only and that numerous changes, modifications and alterations can be employed without departing from the spirit and scope of the invention.

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EXAMPLE I: Cloning of bcl-x

Avian lymphocytes develop in two distinct organs, the bursa of Fabricius and the thymus. B and T cells developing in these organs share a common feature in that cells from both locations undergo the rapid induction of programmed cell death upon removal from the stromal components of the organ (Cooper et al., 1991; Neiman et al., 1991). The inventors used a murine bcl-2 cDNA probe to clone avian bcl-x. The nucleotide sequence of bcl-x (SEQ ID NOS: 1 and 3) displayed low level sequence identity (56%) with bcl-2, and contained an open reading frame which showed significant similarity to the open reading frame found in the unspliced bcl-2b transcript derived from the bcl-2 gene in both humans and mice (Figure 1A and B). Sequencing of a genomic fragment containing bcl-x demonstrated that the 1.3 kb cDNA had also arisen from a linear genomic sequence in the absence of splicing. This feature of the sequences raised the possibility that the bcl-x cDNA may have arisen from an unprocessed pseudogene present within the avian genome.

EXAMPLE II: bcl-x Is Expressed in Many Tissues and Is Highly Conserved in Vertebrate Evolution

Northern blot analysis of various tissue RNA samples isolated from a newly hatched chicken revealed that a bcl-x specific probe hybridized to a 2.7 kb mRNA species present at highest levels in the thymus and central nervous system (Figure 2). In contrast a murine bcl-2-specific probe recognized an mRNA species of approximately 6.5 kb present at roughly equal levels in all tissues assayed.

bcl-x is highly conserved in the chicken, mouse and human genomes. Chicken bcl-x and mouse bcl-2 probes hybridized efficiently to DNA from all three species. However, the bcl-x and bcl-2 probes bound to distinct segments of genomic DNA indicating that they were

recognizing independent sequences, both of which have been highly conserved during vertebrate evolution (Figure 3).

EXAMPLE III: Identification of Two Distinct Human bcl-x cDNAs

The inventors next cloned human homologues of bcl-x. They identified two separate types of human bcl-x cDNAs which contained distinct open reading frames flanked by identical 5' and 3' untranslated sequences. The larger type of cDNA, bcl-x_L (SEQ ID NO:6), contained an open reading frame (SEQ IDNO:7) with greater than 76% nucleotide and 74% amino acid identity (85% amino acid similarity) to chicken bcl-x (SEQ ID NO:2). However, the human bcl-x_L (SEQ ID NO:6) cDNA diverged from the chicken bcl-x (SEQ IDNOS:1 and 3) sequence at a position corresponding to where the two coding exons of bcl-2 are joined to form the bcl-2a transcript and where bcl-2a diverges from bcl-2b. It is the bcl-2a transcript that encodes the functional activities previously ascribed to the bcl-2 gene. From the point of its divergence from the chicken bcl-x sequence, the human bcl-x_L open reading frame (SEQ IDNO:7) extends another 45 amino acids before a termination codon is reached. The first 7 out of 8 of these novel amino acids were identical to amino acids encoded by the second coding exon of bcl-2 and present in the bcl-2a but not the bcl-2b mRNAs of both human and mice (Figure 4a; Tsujimoto et al., 1986; Negrini et al., 1987). The last 36 amino acids encoded by bcl-x_L also showed significant sequence similarity to the hydrophobic domain of bcl-2a thought to play a role in the insertion of the bcl-2 protein into cytoplasmic membranes (Chen-Levy et al., 1989; Chen-Levy and Cleary, 1990). Consistent with the addition of these novel bcl-x_L sequences as a result of mRNA processing, the genomic sequence that encodes the last 45 amino acids of bcl-x_L is found on a separate exon from the exon that encodes the rest of the open reading frame.

The second type of human bcl-x-derived cDNA (Figure 4b) identified, bcl-x_S (SEQ ID NO:8), differs from bcl-x_L (SEQ ID NO:6)

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because it lacks the sequence that encodes a stretch of 63 amino acids present within the bcl-x_L open reading frame (SEQ ID NO:7) (this region is indicated as BCL-X₁ (SEQ IDNO:4) in Figure 4c). This deletion occurs as a result of the splicing of the second coding exon observed in bcl-x_L SEQ 5 ID NO:6) to a more proximal 5' splice donor within the first coding exon. The addition of the 45 amino acids derived from the second coding exon begins precisely at the position of a potential splice donor site, AG/GT, located within the open reading frame of bcl-x_L. The use of this splice donor site in forming the bcl-X_S cDNA results in the deletion of the 63 10 amino acid sequence that displays greatest similarity between bcl-2 and bcl-x_L. This amino acid sequence encoded for by BCL-X_S (SEQ ID NO:9), displays 73% identity with the same region in human BCL-2 (SEQ IDNO:5). This region of BCL-2 is also the most highly conserved region between chicken, murine, and human BCL-2 (Cazals-Hatem et al., 1992; 15 Eguchi et al., 1992).

bcl-x_L and bcl-x_S were transcribed into RNA and then subjected to in vitro translation. As seen in Figure 5, both bcl-x_L and bcl-x_S cDNAs result in translational products of the approximate size predicted by the open reading frames.

20 EXAMPLE IV: **bcl-x_L Can Serve as an Inhibitor of Apoptotic Cell Death**

The murine IL-3-dependent prolymphocytic cell line FL5.12 was transfected with the human bcl-x_L cDNA (SEQ ID NO:6) inserted into the EcoRI cloning site of the pSFFV-Neo expression plasmid. Cells were 25 selected for neomycin resistance for 10 days and then used as a polyclonal population to test their resistance to apoptosis following removal of IL-3. bcl-x_L-transfected cells had similar growth kinetics compared to the parental cell line as well as to neomycin-transfected control cells. For comparison, cells were also transfected with the human 30 bcl-2a open reading frame inserted in the EcoRI cloning site of pSFFV-

Neo. Neomycin-resistant cells were then subjected to IL-3 deprivation, and the number of surviving cells was calculated in triplicate beginning at the time of IL-3 deprivation.

As can be seen in Figure 6, FL5.12 cells transfected with the neomycin construct alone underwent rapid cell death following the removal of the growth factor. Serial examination revealed that these cells underwent apoptosis as manifested by plasma membrane blebbing, cell volume loss, nuclear condensation, and degradation of nuclear DNA at nucleosomal intervals as previously reported (Hockenberry et al., 1990; Nuñez et al., 1990). In contrast, bcl-2-transfected cells demonstrated significant resistance to cell death, and could be readily induced to reenter the cell cycle upon readdition of IL-3. Expression of bcl-2 in greater than 95% of the bulk transfected cells was demonstrated by specific staining with a bcl-2-specific monoclonal antibody.

When stable bcl-x_L transfectants were subjected to IL-3 deprivation they displayed dramatic resistance to cell death with essentially no loss of cell viability over the 8 day culture period. This resistance to cell death was significantly greater than the resistance of bcl-2-transfected cells in which there was a reproducible 50% decrease in surviving cell number over a similar time period (Figure 6). Cotransfection of bcl-2 and bcl-x_L did not improve cell survival beyond that of transfection with bcl-x_L alone. The dramatic survival of bcl-x_L-transfected cells was not due to ongoing cell proliferation as a result of transformation or the induction of growth factor-independent cell proliferation. Following IL-3 deprivation, the cells rapidly took on a quiescent phenotype arresting in a G0/G1 phase of the cell cycle as measured by cell size and DNA content and did not reenter the cell cycle until IL-3 was readded. Readdition of IL-3 led to rapid blast transformation and cell cycle progression. These data indicate that expression of bcl-x_L can lead to significant resistance to apoptotic cell death that is at least as great as that conferred by bcl-2. This property of

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bcl-x_L-transfected cells does not appear to result from cellular transformation that results in IL-3-independent cell growth.

Stable transfection of bcl-x_L prevents apoptotic cell death following growth factor deprivation of an IL-3-dependent cell line to an even greater extent than overexpression of bcl-2. The combination of the two vectors was no better at preventing apoptotic cell death than bcl-x_L alone. This indicates that bcl-x_L plays a major role in regulating the dependence of cells on continuous exogenous signals to prevent cell death.

EXAMPLE V: bcl-xS Can Inhibit the Ability of bcl-2 to Prevent Apoptotic Cell Death.

The bcl-x_S isoform of bcl-x plays a role in the regulation of apoptotic cell death. FL5.12 cells were stably transfected with a human bcl-x_S expression plasmid (Figure 7). Stable transfectants were easily isolated and their expression of bcl-x_S mRNA was confirmed by Northern blot analysis (Fig. 7C). In the presence of IL-3, these cells appeared morphologically normal and displayed growth characteristics indistinguishable from the parental cells or neomycin-transfected controls. Furthermore, the cells died with kinetics indistinguishable from neomycin-transfected control cells upon deprivation of IL-3. bcl-2-transfected cells displayed characteristic resistance to apoptotic cell death upon removal of IL-3. Remarkably, however, when bcl-x_S was cotransfected with bcl-2 and stable transfectants isolated, the cells reacquired sensitivity to growth factor withdrawal, undergoing apoptotic cell death upon IL-3 deprivation. Nevertheless, there was a significant delay in the onset of cell death within this polyclonal population. The sensitivity to IL-3 deprivation of cells co-transfected with bcl-2 and bcl-x_S was not the result of reduced bcl-2 expression since both bulk populations of bcl-2 and bcl-2 + bcl-x_S transfected cells displayed roughly equivalent levels of bcl-2 protein. The magnitude of the ability of bcl-x_S to inhibit bcl-2 function, in

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number of subclones isolated from the co-transfected population of cells was studied. All of these cells expressed high levels of transfected bcl-2, and all demonstrated a reduced resistance to apoptotic cell death upon growth factor withdrawal with some clones demonstrating an almost 5 complete abrogation of bcl-2 function in the presence of bcl-x_s expression (Figure 7b). Furthermore, when assayed at 24 and 48 hours after IL-3 deprivation, DNA from cells co-transfected with bcl-2 and bcl-x_s showed a clear nucleosomal pattern of degradation while DNA from cells transfected with bcl-2 alone or bcl-x_L alone did not. Although there was a 10 correlation between the inhibition of bcl-2 function and the bcl-x_s mRNA levels expressed by the cells, the precise stoichiometry between bcl-x_s expression and bcl-2 functional inhibition was not determined. In subclones which co-express both bcl-2 and bcl-x_s, there is significant 15 inhibition of bcl-2-induced resistance to apoptosis by co-expression of bcl-x_s. The average survival of bcl-2-transfected cells 96 hours after IL-3 removal was 79 + 14 % (mean + 1 S.D., n=3) while the average survival of subclones coexpressing bcl-2 and bcl-x_s was 8 + 8 % (mean + 1 S.D., n=6).

This induction of apoptotic cell death required that the bcl-x_s 20 construct be expressed in the sense orientation, as stable introduction of a pSFFV-Neo plasmid containing bcl-x_s cloned in the antisense orientation had no effect on the ability of bcl-2 to prevent apoptotic cell death upon growth factor deprivation (Figure 8). The stable expression of bcl-x_s had no effect on cell growth in the presence of growth factor or on the rate of 25 apoptotic cell death following growth factor removal. However, bcl-x_s could prevent the ability of stable bcl-2 expression to inhibit apoptotic cell death upon growth factor removal.

These data indicate that the expression of the bcl-x_s isoform of the bcl-x gene likely plays a dominant role in regulating the ability of other 30 growth survival genes such as bcl-2 to prevent apoptotic cell death. bcl-

x_s expression increased the dependence of the cells on exogenous signals such as growth factors to actively prevent cell death.

EXAMPLE VI: Expression of bcl-x During T Cell Development and Activation.

5 The highest level of mRNA for bcl-x in chickens was observed in the organs where lymphoid development takes place. As can be seen in Figure 9, bcl-x mRNA can be readily detected in human thymocytes. Upon fractionation of human thymocytes into immature and mature populations, in the absence of mitogen stimulation bcl-x expression is
10 confined to the immature "double-positive" thymocytes which express both CD4 and CD8. bcl-x mRNA was not detected in unstimulated mature "single positive" (CD4+CD8- and CD4-CD8+) thymocytes or in peripheral blood T cells.

15 Within the thymus, bcl-x was expressed in immature double-positive thymocytes but was not observed in freshly isolated single-positive thymocytes or peripheral blood T cells. The bcl-x mRNA species found in the double-positive thymocytes was almost exclusively of the bcl-x_s form. Previous studies have shown that bcl-2 does not inhibit negative selection that normally occurs at the double-positive stage of
20 thymocyte development (Sentman et al., 1991; Strasser et al., 1991a). The stable expression of bcl-x_s at this stage of development provides a potential explanation for this observation. Nevertheless it has been shown that bcl-2 can prevent some forms of apoptosis that occur in double-positive thymocytes (Sentman et al., 1991; Strasser et al., 1991a;
25 Seigel et al., 1991). This indicates that the influence of bcl-2 relative to bcl-x_s in regulating the central events involved in apoptotic cell death may vary depending on the pathway which initiates the cell death response. Alternatively down-regulation of bcl-x expression at the mRNA or protein level may permit the effects of bcl-2 to predominate. Under these

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conditions it may be possible for over-expression of bcl-2 to prevent apoptotic cell death.

Although single-positive thymocytes and mature peripheral blood T cells fail to express bcl-x mRNA, both populations can be rapidly induced 5 to express high levels of bcl-x mRNA following mitogenic activation. Again the predominant mRNA species observed encodes bcl-x_s (SEQ ID NO:8). This shows that T cell activation induces the expression of bcl-x_s to increase the dependence of the cell on the growth factors provided in its local environment. Activation of peripheral T cells can render them 10 susceptible to apoptosis (Kawabe and Ochi, 1991; Webb et al., 1990), a finding previously at odds with the upregulation of bcl-2 expression observed following T cell activation (Graninger et al., 1987; Reed et al., 1987). The inducible expression of bcl-x_s in these populations may serve 15 to regulate the amplification of T cells involved in an immune response by making them highly dependent on continuous exogenous signaling to prevent their deletion by apoptosis. Thus it appears that the differential regulation of bcl-x during T cell development and T cell activation may play a central role in regulating two important forms of apoptosis occurring in this cell lineage that were previously reported to be regulated 20 independently of bcl-2. bcl-x_s expression plays an important role in the regulation of both developmentally- and activationally-induced cell death.

In addition, there is a significant difference in these populations when these isolated cell populations were stimulated with the mitogenic combination of PMA and ionomycin. Six hour stimulation with PMA and 25 ionomycin had no effect on bcl-x mRNA expression in double-positive thymocyte populations, but induced a dramatic increase in bcl-x mRNA expression in both single-positive thymocytes and peripheral blood T cells. Thus, it is likely that bcl-x mRNA is expressed constitutively in T cells with immature phenotypes that are in the process of undergoing 30 developmental selection. Cells that have completed developmental

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selection down-regulate the expression of bcl-x, but bcl-x expression can be rapidly induced by T cell activation.

bcl-2 has also been reported to be induced upon T cell activation (Graninger et al., 1987; Reed et al., 1987). The kinetics of bcl-x and bcl-
5 2 induction in peripheral blood T cells differed dramatically. Peripheral
blood T cells were isolated at various time points after activation with a
mitogenic combination of PMA and ionomycin (Figure 10). bcl-x was
rapidly induced upon activation with detectable mRNA appearing within
the first 6 hours after stimulation. Thereafter, bcl-x mRNA was expressed
10 at a relatively constant level. In contrast, bcl-2 mRNA was first detected
between 6 and 12 hours after activation and underwent progressive
accumulation over the first 24 hours in culture following mitogen
activation. Similar results were obtained following antigen-receptor
crosslinking. Since the two probes used to detect bcl-x and bcl-2 are of
15 similar size and GC content, the inventors were able to estimate the
differences in the steady state mRNA levels between bcl-x and bcl-2.
There is approximately a 50-fold difference in the steady state
accumulation of bcl-x and bcl-2 mRNA even after 24 hours of cell
activation. Thus, bcl-x mRNA accumulation occurs more rapidly and to a
20 higher steady state level than does the induction of bcl-2 mRNA upon
T cell activation.

EXAMPLE VII: Tissue-Specific Expression of bcl-x_L and bcl-x_S

The present inventors used the polymerase chain reaction (PCR) to quantitate the relative abundance of the bcl-x_L and bcl-x_S mRNAs in a
25 series of RNA samples obtained during T cell development and activation
(Figure 11) (PATENT NO. 4,603,102 INCORPORATED HEREIN BY
REFERENCE). PCR primers that bind to sequences shared by bcl-x_L (SEQ
ID NO:6) and bcl-x_S a(SEQ IDNO:8) and that flank the region that is
deleted in bcl-x_S (position 510 to 698 of SEQ ID NO:6) were used as
30 primers to amplify mRNA following reverse transcription. These two

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primers are located on separate coding exons and therefore the product of any contaminating genomic DNA will be considerably larger than the products of the RNA species of interest. Under the conditions of the PCR reactions, the relative ratios of the two bcl-x mRNA species can be
5 measured as demonstrated in the control study presented in Figure 11B. The inventors determined the expression of bcl-x mRNA species in unfractionated thymocytes and in peripheral blood T cells cultured in media alone or stimulated for 6 hours with PMA and ionomycin. As shown by the Northern blot analyses, resting T cells do not express bcl-x transcripts that can be identified by PCR. In contrast, activated T cells express an easily detectable PCR product comprised predominantly of the bcl-x_S form. Unfractionated thymocytes also express bcl-x_S mRNA almost exclusively. These data show that both activated T cells and double-positive thymocytes selectively express the form of bcl-x that enhances
10 the dependence of the cell on exogenous signals to prevent apoptosis.
15 This finding is consistent with the inability of overexpression of bcl-2 to overcome negative selection during the development of double-positive thymocytes as well as the failure of bcl-2 overexpression to increase significantly T cell numbers during peripheral T cell responses.

20 The other major tissue in chickens that demonstrated a relatively high level of bcl-x expression by Northern blot analysis was the central nervous system. PCR analysis of adult human brain mRNA shows expression exclusively of the bcl-x_L mRNA species (Figure 11A).

25 Thus expression of bcl-x_L is correlated with the ability of adult neural tissue to maintain long term post-mitotic cell viability. Therefore, it appears that different tissues can differentially regulate both the expression and splicing of bcl-x and thus adapt the functional properties of this gene to regulate their relative sensitivity to potential mediators of apoptotic cell death.

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EXAMPLE VIII: Cloning and Construction of Plasmids

Chicken bursal, spleen and thymic cDNA libraries and a genomic library that was made from red blood cells were screened with a murine bcl-2 cDNA at low stringency. The filters were hybridized in Stark's
5 solution (50% formamide, 5x SSC [1x equals 0.15 M NaCl and 0.015 sodium citrate], 1x Denhardt's solution, 24 mM sodium phosphate, pH 6.5, 250 mg of RNA per ml) with 10% dextran sulfate at 42°C overnight. The final wash conditions were 20 minutes at 42°C in 0.1x SSC. Inserts
10 from positive clones were subcloned into pGEM7 (Promega) and sequenced by a dideoxy termination method.

The human bcl-x_s (SEQ ID NO:8) was cloned from a thymic cDNA library using a BamHI/SphI fragment of the chicken bcl-x under the hybridization and washing conditions described above. The insert was then amplified from the plaque purified phage by PCR using Ig11 primers
15 with XbaI linker sites and pfu polymerase. The 0.84 kb amplified fragment was then subcloned into pBluescript-SK+ (Stratagene) for sequence analysis.

To clone the bcl-x_L cDNA (SEQ ID NOS: 1 and 3) and to reclone the bcl-x_s cDNA (SEQ ID NO:8) into a form to be used in functional analysis,
20 PCR primers corresponding to sequences in the 5' untranslated region (5'-TTGGACAATGGACTGGTTGA-3' (5'- end of SEQ ID NO:8)) and the 3' untranslated region (5'-GTAGAGTGGATGGTCAGTG-3' (3'-end of SEQ ID NO:8)) of the bcl-x_s cDNA were synthesized. The primers contained EcoRI linkers for subcloning and were used to amplify clones from cDNA
25 libraries prepared from human T cells, the T cell line Jurkat, and human brain. The phage (10⁷ pfu) were boiled for 5 minutes in 25 ml of water prior to the PCR reactions (1.25 minutes at 94°C, 2 minutes at 56°C, 3 minutes at 72°C x 35 cycles). Bands of appropriate size (0.8 kb for bcl-x_L and 0.6 kb for bcl-x_s) that could hybridize to the bcl-x_s cDNA (SEQ ID NO:8) were subcloned into the EcoRI site of pBluescript-SK+ for

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sequence analysis and production of in vitro transcription and translation products. For transfection and subsequent functional assays, the bcl-x_L (SEQ ID NO:6) and bcl-x_S (SEQ ID NO:8) inserts were excised from pBluescript SK+ and subcloned into the EcoRI site of pSFFV-Neo (Neo; Fuhlbrigge et al., 1988). Orientation of the inserts was determined by restriction enzyme mapping and plasmids with inserts in the forward orientation were designated pSFFV-Neo-bcl-x_L (bcl-x_L) and pSFFV-Neo-bcl-x_S (bcl-x_S), while plasmids with inserts in the reverse orientation were named pSFFV-Neo-bcl-x_Lrev (bcl-x_Lrev) and pSFFV-Neo-bcl-x_Srev (bcl-x_Srev).

Sequence comparisons and peptide analyses were performed with the University of Wisconsin Genetics Computer Group programs: FASTA, TFASTA, PILEUP, PEPTIDESTRUCTURE, and GAP. The nucleotide sequences of chicken bcl-x (SEQ ID NOS:1 and 3), human bcl-x_L (SEQ ID NO:6), and human bcl-x_S (SEQ ID NO:8) have been sent to the GenBank database.

EXAMPLE IX: Southern Blot Analysis

Genomic DNA from chicken mouse, and human lymphoid cells was isolated by standard methods, as previously described (Thompson and Neiman, 1987). The DNA was quantitated and 10 mg was digested with indicated restriction enzymes overnight. Digested DNA was separated on a 1% agarose gel and blotted onto nitrocellulose. Blots were hybridized as described above with either an SphI/BamHI fragment of chicken bcl-x (SEQ ID NOS: 1 and 3) or a HindIII/BamHI fragment from the first coding exon of mouse bcl-2. Washing conditions were as described above.

EXAMPLE X: Cell Isolation and RNA Analysis

For the chicken tissue Northern blots, newly hatched chicks were sacrificed and RNA was isolated from the indicated tissues by a guanidinium isothiocyanate method followed by centrifugation through a

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cesium chloride gradient as previously described (Thompson et al., 1986). RNA was equalized to the 28S ribosomal RNA, separated on agarose/formaldehyde gels and subsequently blotted onto nitrocellulose. The blots were probed with the SphI/BamHI chicken bcl-x fragment. Blots 5 were then stripped by boiling and hybridized with the HindIII/BamHI mouse bcl-2 probe.

Human T cells were isolated from healthy donors by leukophoresis, followed by density gradient centrifugation. CD28-positive T cells were negatively selected via an immunomagnetic procedure (June et al., 1987). 10 RNA was then isolated from T cells that were either resting or activated with phorbol myristate acetate (PMA; 10 ng/ml) and ionomycin (0.8 mg/ml) for the indicated times. RNA was equalized and subjected to electrophoresis through agarose/formaldehyde denaturing gels and the gels were then blotted as described above. Duplicate blots were probed 15 with the human bcl-x_s cDNA or the mouse bcl-2 exon II fragment and a human HLA class I cDNA. Final washing conditions were 0.1xSSC, 0.1% SDS for 20 minutes at 56°C.

Human thymocytes were isolated from surgical pathology specimens from children under the age of three who had undergone 20 cardiothoracic surgery. Thymic tissue was passed through nylon mesh to obtain a single cell suspension followed by separation of mononuclear cells on a ficoll-hypaque cushion. RNA was then extracted from either resting or PMA and Ionomycin stimulated cells and subjected to Northern blot analysis. To determine if bcl-x expression was regulated during 25 thymocyte development, thymocytes were separated into mature ("single-positive" cells) and immature ("double-positive") subpopulations prior to stimulation and RNA extraction as previously described (Turka et al., 1991). Thymocyte fractionation was performed by negative immunomagnetic selection. Immature thymocytes were prepared by 30 removal of cells expressing high levels of CD28, while mature thymocytes

were selected by the removal of CD1+ cells. The expression status of the resulting cells for CD4 and CD8 confirmed that greater than 90% of the immature population expressed CD4 and CD8 while greater than 95% of the mature population were single-positive cells.

5 To determine which form of bcl-x mRNA was being expressed, 1 mg of total cellular RNA or 0.1 mg of polyA+ RNA from the various sources was reversed transcribed with AMV reverse transcriptase for 1 hour at 42°C. Twenty percent of the cDNA product was then subjected to PCR using the 5' and 3' primers and amplification conditions described
10 above. Because the two primers are each located on separate coding exons, the amplification product of contaminating genomic DNA will be much larger than the product of either of the two RNA species, bcl-x_L and bcl-x_S. To assure that the PCR conditions were not biased to one form of the cDNA, concurrent PCR reactions were run with plasmids containing
15 bcl-x_L or bcl-x_S alone or mixed in various ratios.

EXAMPLE XI: Cell Transfection and Functional Studies

Murine FL5.12 cells were cultured as previously described (Hockenberry et al., 1990; Nuñez et al., 1990). Cells were transfected by electroporation (200V, 960mF) with the pSFFV-Neo plasmid containing either bcl-2a (bcl-2), bcl-x_L in both transcriptional orientations (bcl-x_L and bcl-x_Lrev), and bcl-x_S in both transcriptional orientations (bcl-x_S and bcl-x_Srev). As a control, transfections were also performed with the pSFFV-Neo plasmid without an insert (Neo). Transfectants were selected for the acquisition of neomycin resistance by growth in the presence of G418 (1 mg/ml). Bulk transfectants and single cell clones (generated by limiting dilution) were maintained by growth in media supplemented with IL-3 as previously described (Nuñez et al., 1990). To assess cell survival, cells were first grown at 2 x 10⁵/ml in the presence of an optimal concentration of growth factor for 20-24 hours. The cells were then
25 washed with RPMI 1640 medium three times to remove any residual IL-3,
30

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and plated at 105 cells per well in 96 well culture dishes in medium supplemented with 10% fetal calf serum. Cell survival was determined at the indicated time points by trypan blue exclusion. To confirm that cell death was due to apoptosis, transfected cells (2x10⁶) were isolated 0, 8, 5 24, and 48 hours after growth factor removal and lysed in 1.0% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, and 200 mg/ml proteinase K for 2 hours at 50°C. Following incubation, samples were treated with RNase A (20 mg/ml) for 2 hours at 37°C, phenol/chloroform extracted, and ethanol precipitated. DNA was then separated on a 1.2% agarose gel and stained with ethidium bromide.

BCL-2 specific antibody did not cross react with BCL-X. Cells were washed and fixed with 1% paraformaldehyde for 10 minutes at room temperature, then stained with the 6C8 monoclonal antibody, a hamster monoclonal antibody specific for human bcl-2 (Hockenberry et al., 1990), 15 or an isotype-matched hamster antibody control in 0.3% saponin in PBS for 30 minutes at 4°C. Cells were washed in 0.03% saponin/PBS and incubated with biotinylated F(ab')2 goat anti-hamster IgG for 30 minutes at 4°C. Cells were washed in 0.03% saponin/PBS and incubated with RED 670-streptavidin and analyzed by flow cytometry. Analysis of bcl-x_L- 20 and bcl-x_S-transfected cells showed that the bcl-2-specific antibody did not crossreact with the BCL-X expressed in these cells. bcl-x expression was confirmed by Northern blot analysis, with b-actin expression utilized as a loading control.

EXAMPLE XII: In vitro Transcription and Translation of bcl-x

pBluescript-SK+ plasmids containing bcl-x_L (SEQ ID NO:6) and bcl-x_S (SEQ ID NO:8) were linearized at the 3' multiple cloning site with XbaI and BamHI respectively and transcribed with T7 RNA polymerase for 1 hour at 37°C. bcl-x_L was also linearized at the 5' multiple cloning site with XhoI and transcribed with T3 polymerase as an antisense control. 30 The resulting run-off transcripts (bcl-x_L, bcl-x_S and bcl-x_S-as) were

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phenol/chloroform extracted and ethanol precipitated. In vitro translation was then performed with a rabbit reticulocyte lysate kit (Promega) in the presence of ^{35}S -methionine for 1 hour at 30°C. Five ml of lysate was added to SDS loading buffer and subjected to SDS-PAGE (15% gel). Gels were dried and exposed to x-ray film.

5

Because numerous modifications and variations in the practice of the present invention are expected to occur to those skilled in the art, only such limitations as appear in the appended claims should be placed thereon.

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SEQUENCE LISTING

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10 (ii) TITLE OF INVENTION: Vertebrate Apoptosis Gene:
Compositions and Methods

(iii) NUMBER OF SEQUENCES: 9

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS, ASCII

25 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: UNKNOWN
(B) FILING DATE: CONCURRENTLY FILED
(C) CLASSIFICATION: UNKNOWN

30 (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/081.448
(B) FILING DATE: 22 JUNE 1993

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: PARKER, David L.
(B) REGISTRATION NUMBER: 32,165
35 (C) REFERENCE/DOCKET NUMBER: ARCD090

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(2) INFORMATION FOR SEQ ID NO:1:

40 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1274 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 179..751

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG TCC AGC AGT AAC CGG GAG TTA GTG ATT GAC TTT GTC TCC TAC AAG 226
Met Ser Ser Asn Arg Glu Leu Val Ile Asp Phe Val Ser Tyr Lys 10
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CTC TCG CAG AGG GGG CAC TGC TGG AGC GAG CTG GAG GAA GAG GAT GAG 274
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418									
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TAC	CGG	AGG	GCT	TTC	AGC	GAC	CTC	ACC	CCT
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GAT	GGT	GTG	AAC	TGG	GGG	CGC	ATC	GTC	TTC
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Q

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 AGCCCTTGT CTTGGGCATG TTCCCTCATGT CATTAAACAG CAGGGAGTGG AGCTTCCTCC 1118
 CCTCCCGTGT CAGCAGTGT CCAGGCCGGC CCTGTGATCT GTGGGGTAA CAGCTACTTC 1178
 TTCAATTCTGG AGATGGGACG ATGTCTGGCG CTGCCATGCC GTGGAGTGGAA TCCTGCAGCA 1238
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(2) INFORMATION FOR SEQ ID NO:2:

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 190 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 1 5 10 15
 Leu Ser Gln Arg Gly His Cys Trp Ser Glu Leu Glu Glu Asp Glu
 20 25 30

10
20

Asn	Arg	Thr	Asp	Thr	Ala	Ala	Glu	Ala	Glu	Met	Asp	Ser	Val	Leu	Asn	
35															45	
Gly	Ser	Pro	Ser	Trp	His	Pro	Pro	Ala	Gly	His	Val	Val	Asn	Gly	Ala	
															50	
5	Thr	Val	His	Arg	Ser	Ser	Leu	Glu	Val	His	Glu	Ile	Val	Arg	Ala	Ser
															60	
															65	
															70	
															75	
															80	
															85	
															90	
															95	
10	Tyr	Arg	Arg	Ala	Phe	Ser	Asp	Leu	Thr	Ser	Gln	Leu	His	Ile	Thr	Pro
															100	
															105	
															110	
															115	
															120	
															125	
															130	
															135	
															140	
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															145	
															150	
															155	
															160	
															165	
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															190	

(2) INFORMATION FOR SEQ ID NO:3:

(1) SEQUENCE CHARACTERISTICS:

69 1

29

(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGAGCGGTGC ACCCAGCGCG CAGGAATTG

(2) INFORMATION FOR SEQ ID NO:4:

10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Val Val Asn Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile Val
1 5 10 15

Ala Phe Phe Ser Phe Gly Gly Ala Leu Cys Val Glu Ser Val Asp Lys
20 25 30

Glu Met Gln Val Leu Val Ser Arg Ile Ala Ala Trp Met Ala Thr Tyr
35 40 45

Leu Asn Asp His Leu Glu Pro Trp Ile Gln Glu Asn Gly Gly Trp
50 55 60

5

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92

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 205 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met	10	
1	5	15
Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala	20	25
		30
Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile	35	40
		45
Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp	50	55
		60
Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala	65	70
		75
Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala	85	90
		95
Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe	100	105
		110

43

Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly
 115 120 125
 Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp GLY Val Asn Trp
 130 135 140
 GLY Arg Ile Val Ala Phe Phe Glu Gly Gly Val Met Cys Val Glu
 145 150 155 160
 Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp
 165 170 175
 Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn
 180 185 190
 GLY GLY Trp Val Gly Ala Ser Gly Asp Val Ser Leu GLY
 195 200 205

10

(2) INFORMATION FOR SEQ ID NO:6:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 926 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: DNA (genomic)
 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 135..836
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
 20

4

	GAATCTCTTT	CTCTCCCTTC	AGAATCTTAT	CTTGGCTTG	GATCTTAGAA	GAGAATCACT	60
	AACCAGAGAC	GAGACTCAGT	GAGTGAGCAG	GTGTTTGGAA	CAATGGACTG	GTGAGCCCCA	120
5	TCCCTATTAT	AAAATG	TCTCAG	AGCAAC	CGGCTG	GTCGTT	170
	Met	Ser	Gln	Ser	Asn	Arg	
	1	5	9	13	17	Leu	
	CTC	TCC	TAC	AAG	CTT	TCC	218
	Leu	Ser	Tyr	Lys	Ile	Ser	
	15	19	23	27	31	Arg	
	GAT	GTG	GAA	GAG	AAC	AGG	266
	Asp	Val	Glu	Glu	Glu	Thr	
	30	34	38	42	46	Ala	
	ATG	GAG	ACC	CCC	AGT	GCC	314
	Met	Glu	Thr	Pro	Ser	ATC	
	45	49	53	57	61	AAT	
	GAC	AGC	CCC	GGC	ATC	GGC	362
	Asp	Ser	Pro	Ala	Ile	Asn	
	65	69	73	77	81	Gly	
	GCC	GGG	GAG	GTG	AAT	GGAA	410
	Ala	Arg	Glu	Val	Ile	Pro	
	80	84	88	92	96	Met	
	GCA	GCG	GAC	GAG	TTT	GAA	458
	Ala	Gly	Asp	Glu	CCTG	CGG	
	95	99	103	107	111	TAC	
	ACA	TCC	CAG	CTC	CAC	ACC	506
	Thr	Ser	Gln	Ile	Leu	His	
	15	19	23	27	31	Arg	
	CTG	CGG	CGG	CGG	CGG	CGG	
	100	104	108	112	116	120	
	AGT	AGC	AGC	AGC	AGC	AGC	
	105	109	113	117	121	125	
	GAC	TTC	TTC	TTC	TTC	TTC	
	110	114	118	122	126	130	
	CTG	AGT	AGT	AGT	AGT	AGT	
	115	119	123	127	131	135	
	GAA	GAA	GAA	GAA	GAA	GAA	
	120	124	128	132	136	140	
	TAT	CAG	AGC	AGC	AGC	AGC	
	125	129	133	137	141	145	
	GAA	GAA	GAA	GAA	GAA	GAA	
	130	134	138	142	146	150	

95

96

(2) INFORMATION FOR SEO ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 233 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Ser Gln ser Asn Arg Glu Leu Val Val Asp Phe Leu Ser Tyr Lys
1 5 10 15

Leu Ser Gln Lys Gly Tyr Ser Trp Ser Gln Phe Ser Asp Val Glu Glu
20 25 30

Asn Arg Thr Glu Ala Pro Glu Gly Thr Glu Ser Glu Met Glu Thr Pro
 35 40 45

Ser Ala Ile Asn Gly Asn Pro Ser Trp His Leu Ala Asp Ser Pro Ala
50 55 60

Phe Cys Leu Arg Tyr Arg Arg Ala Phe Ser Asp Leu Thr Ser Gln Leu
100 105 110

97

Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile Val Ala Phe Phe
 130 135 140
 Ser Phe Gly Gly Ala Leu Cys Val Glu Ser Val Asp Lys Glu Met Gln
 145 150 155 160
 5 Val Leu Val Ser Arg Ile Ala Ala Trp Met Ala Thr Tyr Leu Asn Asp
 165 170 175
 His Leu Glu Pro Trp Ile Gln Glu Asn Gly Gly Trp Asp Thr Phe Val
 180 185 190
 10 Glu Leu Tyr Gly Asn Asn Ala Ala Glu Ser Arg Lys Gly Gln Glu
 195 200 205
 Arg Phe Asn Arg Trp Phe Leu Thr Gly Met Thr Val Ala Gly Val Val
 210 215 220
 Leu Leu Gly Ser Leu Phe Ser Arg Lys
 225 230

15 (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 737 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS

(B) LOCATION: 135..647

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

	GAATCTCTT	CTCTCCCTTC	AGAATCTTAT	CTTGCTTTG	GATCTTAGAA	GAGAATCACT	60
	AACCAGAGAC	GAGACTCAGT	GAGTGAGCAG	GTGTGACTG	CAATGGACTG	GTGAGGCCA	120
5	TCCCTATTAT	AAAATG	TCTCAGC	AACCGG	GAGCTG	GTGTTGAC	170
	Met	Ser	Gln	Ser	Asn	Arg	
	1	5	9	13	17	Leu	
	CTC	TCC	TAC	AAG	CTT	TCC	218
	Leu	Ser	Tyr	Lys	Leu	Ser	
10	15	19	23	27	31	Arg	
	GAT	GTG	GAA	GAG	AAC	AGG	266
	Asp	Val	Glu	Glu	Glu	Asn	
	30	34	38	42	46	Pro	
	ATG	GAG	ACC	CCC	AGT	GCC	314
	Met	Glu	Thr	Pro	Ser	ATC	
15	45	49	53	57	61	AAT	
	GAC	AGC	CCC	GGC	GTG	AAT	362
	Asp	Ser	Pro	Asn	Val	Gly	
	65	69	73	77	81	Ala	
	GCC	CGG	GAG	ATC	CCC	ATG	410
	Ala	Arg	Glu	Ile	Val	Met	
20	80	84	88	92	96	Ala	
	GCA	GCG	GAC	TTC	GAA	CTG	458
	Ala	Gly	Asp	Glu	Phe	Leu	

(x)

99

	95	100	105	
	ACA TCC CAG CTC CAC ATC ACC CCA GGG ACA GCA TAT CAG AGC TTT GAA			
	Thr Ser Gln Leu His Ile Thr Pro Gly Thr Ala Tyr Gln Ser Phe Glu			
	110	115	120	506
5	CAG GAT ACT TTT GTG GAA CTC TAT GGG AAC AAT GCA GCA GCC GAG AGC			
	Gln Asp Thr Phe Val Glu Leu Tyr Gly Asn Asn Ala Ala Glu Ser			
	125	130	135	140
10	CGA AAG GGC CAG GAA CGC TTC AAC CGC CGC TGG TTC CTG ACG GGC ATG ACT			
	Arg Lys Gly Gln Glu Arg Phe Asn Arg Trp Phe Leu Thr Gly Met Thr			
	145	150	155	602
	GTG GCC GGC GTG GTT CTG CGC TCA CTC TTC AGT CGG AAA TGACAGACA			
	Val Ala Gly Val Val Leu Leu Gly Ser Leu Phe Ser Arg Lys			
	160	165	170	654
	CTGACCATCC ACTCTAACCT CCCACCCCT TCTCTGCTCC ACCACATCCT CCGTCCAGCC			714
15	GCCATTGCCA CCAGGAGAAC CCG			737

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 170 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

100

Met Ser Gln Ser Asn Arg Glu Leu Val Val Asp Phe Leu Ser Tyr Lys
1 5 10 15
Leu Ser Gln Lys Gly Tyr Ser Trp Ser Gln Phe Ser Asp Val Glu Glu
20 25 30
Asn Arg Thr Glu Ala Pro Glu Gly Thr Glu Ser Glu Met Glu Thr Pro
35 40 45
Ser Ala Ile Asn Gly Asn Pro Ser Trp His Leu Ala Asp Ser Pro Ala
50 55 60
Val Asn Gly Ala Thr Gly His Ser Ser Ser Leu Asp Ala Arg Glu Val
65 70 75 80
Ile Pro Met Ala Ala Val Lys Gln Ala Leu Arg Glu Ala Gly Asp Glu
85 90 95
Phe Glu Leu Arg Tyr Arg Ala Phe Ser Asp Leu Thr Ser Gln Leu
100 105 110
His Ile Thr Pro Gly Thr Ala Tyr Gln Ser Phe Glu Gln Asp Thr Phe
115 120 125
Val Glu Leu Tyr Gly Asn Asn Ala Ala Ala Glu Ser Arg Lys Gly Gln
130 135 140
Glu Arg Phe Asn Arg Trp Phe Leu Thr Gly Met Thr Val Ala Gly Val
145 150 155 160
Val Leu Leu Gly Ser Leu Phe Ser Arg Lys
165 170

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CLAIMS

1. An isolated and purified polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death.

5 2. The isolated and purified polynucleotide of claim 1, wherein said polynucleotide is a DNA molecule.

3. The DNA molecule of claim 2, wherein said encoded polypeptide is BCL-X_L (SEQ ID NO:7), BCL-X_s (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4).

10 4. The DNA molecule of claim 2, wherein said encoded polypeptide comprises the amino acid residue sequence of SEQ ID NOS:2, 4, 7, or 9.

15 5. The isolated and purified polynucleotide of claim 1, wherein said polynucleotide comprises the nucleotide base sequence of SEQ ID NOS: 1 and 3, 6 or 8.

20 6. An isolated and purified polynucleotide comprising a base sequence that is identical or complementary to a segment of at least 10 contiguous bases of SEQ ID NOS: 1 and 3, SEQ ID NO:6, or SEQ ID NO:8 wherein said polynucleotide hybridizes to a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death.

7. An isolated and purified polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death.

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8. The polypeptide of claim 7, wherein the polypeptide is BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4).

9. The polypeptide of claim 7 that comprises the amino acid residue sequence of SEQ ID NO:2, 4, 7, or 9.

5 10. An expression vector comprising a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death.

11. The expression vector of Claim 10, wherein the polynucleotide is operatively linked to an enhancer-promoter.

10 12. A recombinant host cell transfected with a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death.

15 13. The recombinant host cell of claim 12 where the polynucleotide is under the transcriptional control of regulatory signals functional in the recombinant host cell, wherein the regulatory signals appropriately control expression of said polypeptide in a manner to enable all necessary transcriptional and post-transcriptional modification.

20 14. A process of preparing a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death comprising:

a) transfecting a cell with a polynucleotide that encodes the polypeptide to produce a transformed host cell; and

b) maintaining the transformed host cell under biological conditions sufficient for expression of the polypeptide.

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15. An antibody immunoreactive with a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death.

16. The antibody of claim 15 wherein said polypeptide is BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4).

17. A process of detecting a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death, wherein the process comprises immunoreacting the polypeptide with the antibody of claim 15 to form an antibody-polypeptide conjugate and detecting the conjugate.

18. The process of claim 17 wherein said polypeptide is BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4).

19. A process of detecting a messenger RNA transcript that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death, wherein the process comprises hybridizing the messenger RNA transcript with a polynucleotide sequence that encodes the polypeptide to form a duplex and detecting the duplex.

20. The process of claim 19 wherein said polypeptide is BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4).

21. A process of screening a substance for its ability to alter programmed cell death, said process comprising the steps of:

a) providing a cell that contains a functional polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death;

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b) testing the ability of said substance to alter programmed cell death.

22. The process of claim 21 wherein said polypeptide is BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4).

5 23. The process according to claim 21 wherein providing is transforming a host cell with a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death to form a transformed cell and maintaining said transformed cell under biological conditions sufficient for expression of
10 said polypeptide.

24. A process of altering programmed cell death in a cell comprising

15 (a) delivering to the cell an effective amount of a DNA molecule comprising a polynucleotide that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that inhibits or promotes vertebrate programmed cell death; and
 (b) maintaining the cell under conditions sufficient for expression of said polypeptide.

20 25. The process of claim 24 wherein said polypeptide is BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4).

26. The process of claim 24 wherein delivering is injecting the DNA molecule into the cell.

25 27. The process of claim 24, wherein delivering is administering the DNA molecule into the circulatory system of a subject in which the cell is located.

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28. The process of claim 27 wherein administering comprises the steps of:

- (a) providing a vehicle that contains the DNA molecule; and
- (b) administering the vehicle to the subject.

5 29. The process according to claim 28 wherein the vehicle is a vehicle cell transformed or transfected with said DNA molecule or a transfected cell derived from such a transformed or transfected cell.

10 30. The process according to claim 28 wherein the vehicle is a virus or an antibody that specifically infects or immunoreacts with an antigen of the cell.

31. The process according to claim 30 wherein the virus is a retrovirus.

15 32. The process of claim 31 wherein said retrovirus is a vaccinia virus, a picornavirus, a coronavirus, a togavirus, or a rhabdovirus altered in such a way as to render the virus non-pathogenic.

33. The process of claim 24 wherein said cell is a neuronal cell.

34. The process of claim 24 wherein said cell is a lymphocyte.

35. The cell of claim 34 wherein said lymphocyte is a CD4 cell.

20 36. The process of claim 33 further comprising introducing the vector into the cells undergoing or likely to undergo programmed cell death by a process comprising transplanting cells of a multipotent neural cell line into a region of the central nervous system in which said neuronal cells undergoing or likely to undergo programmed cell death are located.

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37. The process of claim 33 further comprising introducing the vector into neuronal cells of an animal by injection of the vector at the site of the peripheral nerve endings of the neuronal cells undergoing or likely to undergo cell death.

5 38. The process of claim 33 further comprising introducing the vector into neuronal cells in culture likely to undergo or undergoing cell death by incubation of the vector with the neuronal cells.

39. A process of preventing or treating programmed cell death in neuronal cells, the process comprising:

10 (a) preparing a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death;

(b) combining the polypeptide with a physiologically acceptable carrier to form a pharmaceutical composition; and

(c) administering the composition to neurons likely to undergo or
15 undergoing programmed cell death.

40. The process of claim 39 wherein the polypeptide is BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4).

41. A process of delivering a gene that encodes a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death for gene therapy, the process comprising:

20 (a) providing the vector of claim 10;

(b) combining the vector with a physiologically acceptable carrier to form a pharmaceutical composition; and

(c) administering said pharmaceutical composition so that the
25 vector will reach the intended cell targets.

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42. The process of claim 41 wherein said pharmaceutical composition is introduced by injection into an animal at the site of said cell targets.

5 43. The process of claim 41 wherein the polypeptide is BCL-X_L (SEQ ID NO:7), BCL-X_S (SEQ ID NO:9) or BCL-X_I (SEQ ID NO:4).

44. The process of claim 41 wherein said cell targets are in the central nervous system and the pharmaceutical composition is introduced by injection into an animal at the site of the peripheral nerve ending which originate from neurons located at the site of said cell targets.

10 45. A process of treating tumorigenic diseases, the process comprising:

- (a) providing an expression vector according to claim 10;
- (b) combining the vector with a physiologically acceptable carrier to form a pharmaceutical composition; and
- 15 (c) administering the composition to tumor cell targets.

46. A process of preventing or treating programmed cell death in lymphocytes, the process comprising:

- (a) preparing a polypeptide other than BCL-2 (SEQ ID NO:5) that promotes or inhibits programmed vertebrate cell death;
- 20 (b) combining the polypeptide with a physiologically acceptable carrier to form a pharmaceutical composition; and
- (c) administering the composition to lymphocytes likely to undergo or undergoing programmed cell death.

Fig. - 1A

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bcl-x	1	MSSSNRELVIDFVSYKLSQRGHCWSELEEDENRTDTAAEAEMD	44
bcl-2 β	1	MAEAGRITGYDNREIVMKYIHYKLSQRGYEW....DAGDVGAAPPGAAPAP	46
bcl-x	45	SVLNGSPSWHPPAGHVUNGATVHRSSLLEVHEIVRASD.....VRQ	84
bcl-2 β	47	GISSQPG.KTPHPAASRDGPARTSPLQTPAAPGAAAGPALSPVPPVVHL	95
bcl-x	85	ALRDAGDEFELRYRRAFSDLTSQLHITPGTAYQSFEQVVNELFHDGVNWG	134
bcl-2 β	96	ALRQAGDDFSRRYRGDFAEMSSQLHLPFTARGRFATVVEELFRDGVNWG	145
bcl-x	135	RIVAFFSGGALCVESVDKEMRVLVGRIVSWMTTYLTIDHLDPWIQENGW	184
bcl-2 β	146	RIVAFFEGGVMCVESVNREMSPLVNDIALWMTEYLNRHLHTWIQDNGW	195
bcl-x	185	VRTALP.... 190	
bcl-2 β	196	VGASGDVSLG 205	

Fig. 1B

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Brain Eye Gizzard Heart Kidney Limb Liver Lung Thymus

bcl-x



bcl-2



Fig. 2

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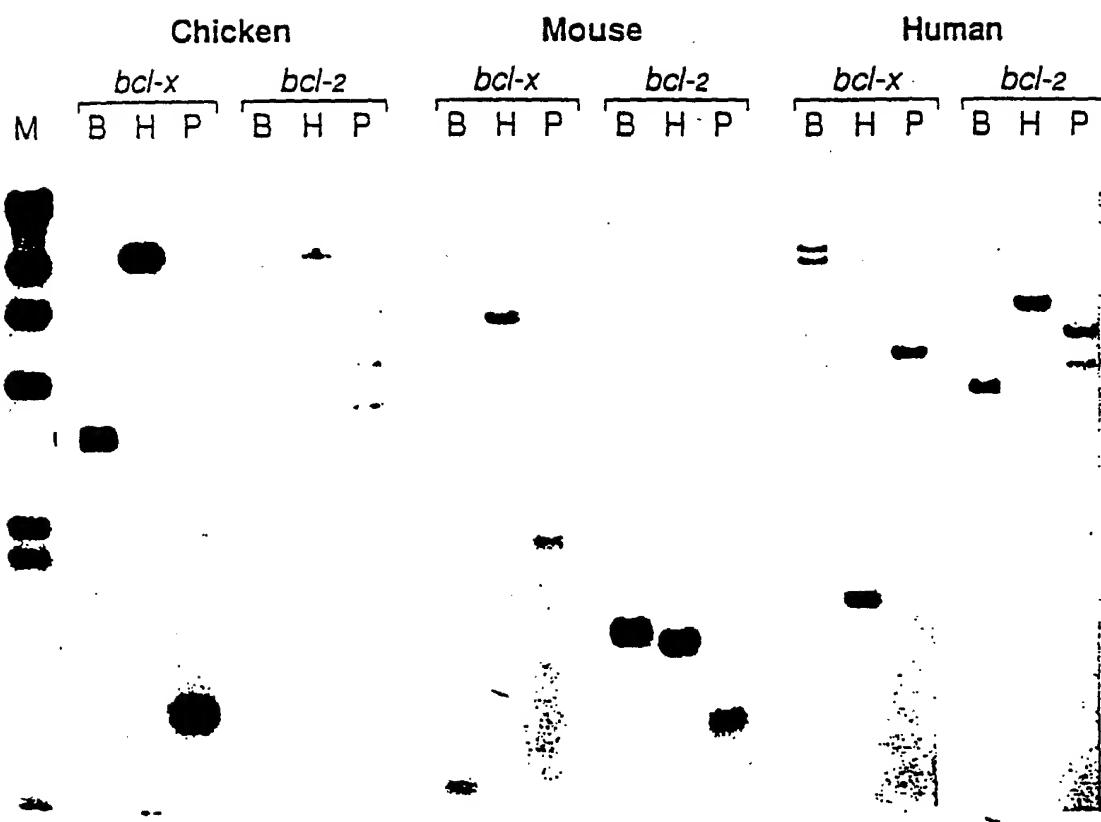


Fig. 3

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Human bcl-xL nucleotide and protein sequence

```

1  gaatctttcttccttcagaatcttatcttggcttggatcttagaagagaatcactaaccagagacg  71
72 agactcagtgaggcagggtttggacaatggactgggtgagccatccctattataaaaatgtctcag 143
               MetSerGln
144 agcaaccgggagctggtggtgactttcttcctacaagcttcccagaaaggatacagctggagtcat 215
               SerAsnArgGluLeuValValAspPheLeuSerTyrLysLeuSerGlnLysGlyTyrSerTrpSerGlnPhe
216 agtgatgtggaagagaacaggactgaggccccagaaggactgaatcgagatggagaccccccagtgccatc 287
               SerAspValGluGluAsnArgThrGluAlaProGluGlyThrGluSerGluMetGluThrProSerAlaIle
288 aatggcaacccatcctggcacctggcagacagccccgcggtaatggagccactgcgcacagcagcagttg 359
               AsnGlyAsnProSerTrpHisLeuAlaAspSerProAlaValAsnGlyAlaThrAlaHisSerSerLeu
360 gatgccgggagggtatccccatggcagcagtaaagcaagcgctgaggcaggcagcagttgaactg 431
               AspAlaArgGluValIleProMetAlaAlaValLysGlnAlaLeuArgGluAlaGlyAspGluPheGluLeu
432 cggtaccggggggcattcagtgacctgacatcccagctccacatccccagggacagcatatcagagctt 503
               ArgTyrArgArgAlaPheSerAspLeuThrSerGlnLeuHisIleThrProGlyThrAlaTyrGlnSerPhe
504 gaacaggttagtgaatgaactttccggatgggtaaactgggtcgcatgtggcttttccttcggc 575
               GluGlnValValAsnGluLeuPheArgAspGlyValAsnTrpGlyArgIleValAlaPheSerPheGly
576 gggcactgtgcgtggaaagcgtagacaaggagatgcaggtattggtgagtccggatcgccagttggatgcc 647
               GlyAlaLeuCysValGluSerValAspLysGluMetGlnValLeuValSerArgIleAlaAlaTrpMetAla
648 acttacctgaatgaccacctagagccttggatccaggagaacggcggctggatactttgtgaaactctat 719
               ThrTyrLeuAsnAspHisLeuGluProTrpIleGlnGluAsnGlyGlyTrpAspThrPheValGluLeuTyr
720 gggacaatgcagcagccgagagccaaaggccaggaacgcgttcaaccgtggcttcgtacggcatgact 791
               GlyAsnAsnAlaAlaGluSerArgLysGlyGlnGluArgPheAsnArgTrpPheLeuThrGlyMetThr
792 gtggccggcgtggctgtggctcaactttcagtccggaaatgaccagacactgaccatccacttaccc 863
               ValAlaGlyValValLeuLeuGlySerLeuPheSerArgLys
864 cccaccccccctctgtccaccacatccgtccagccgcattgcaccaggagaaccccg 926

```

Fig. 4A

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Human bcl-xS nucleotide and protein sequence

1 gaatctttctcccttcagaatcttatcttggtttggatcttagaagagaatcactaaccagagacg 71
72 agactcagtgagtgagcagggtgtttggacaatggactgggtgagcccattttataaaaaatgtctcag 143
MetSerGln
144 agcaaccgggagctgggtggttgactttctcctacaagcttcccagaaaggatacagctggagtcat 215
SerAsnArgGluLeuValValAspPheLeuSerTyrLysLeuSerGlnLysGlyTyrSerTrpSerGlnPhe
216 agtgatgtggaagagaacaggactgaggccccagaaggactgaatcgagatggagaccccatgc 287
SerAspValGluGluAsnArgThrGluAlaProGluGlyThrGluSerGluMetGluThrProSerAlaIle
288 aatggcaacccatctggcacctggcagacagcccccggtgaatggagccactgcccacaggcagttg 359
AsnGlyAsnProSerTrpHisLeuAlaAspSerProAlaValAsnGlyAlaThrAlaHisSerSerLeu
360 gatgcccgagggtgatcccatggcagcagtaaagcaagcgcgtgagggaggcaggcagttgactg 431
AspAlaArgGluValIleProMetAlaAlaValLysGlnAlaLeuArgGluAlaGlyAspGluPheGluLeu
432 cggtaccggcggcattcagtgacctgacatcccagctccacatccccaggacagcatatcagagctt 503
ArgTyrArgArgAlaPheSerAspLeuThrSerGlnLeuHisIleThrProGlyThrAlaTyrGlnSerPhe
504 gaacaggatactttgtggactctatggaaacaatgcagcagccgagagccgaaaggccaggaacgc 575
GluGlnAspThrPheValGluLeuTyrGlyAsnAsnAlaAlaGluSerArgLysGlyGlnGluArgPhe
576 aaccgcgtggcctgacggcatgactgtggccggcgtggctctgctggctcactcttcagtcggaaatga 647
AsnArgTrpPheLeuThrGlyMetThrValAlaGlyValValLeuLeuGlySerLeuPheSerArgLys
648 ccagacactgaccatccactctaccctcccaccccttctgtccaccacatcctccgtccagccgcat 719
720 tgccaccaggagaacccg 737

Fig. 4B

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10

bcl-x_L MSQS
bcl-x_S MSQS

bcl-x_L NRELVVDFLS YKLSQKGYSW SQFSDVEENR TEAPEGTESE METPSAINGN
bcl-x_S NRELVVDFLS YKLSQKGYSW SQFSDVEENR TEAPEGTESE METPSAINGN

bcl-x_L PSWHLADSPA VNGATAHSSS LDAREVIPMA AVKQALREA GDEFELRYRR
bcl-x_S PSWHLADSPA VNGATAHSSS LDAREVIPMA AVKQALREA GDEFELRYRR

bcl-x_L AFSDLTSQLH ITPGTAYQSF EQVNVNLF RD GVNWGRIVAF FSFGGALCVE
bcl-x_S AFSDLTSQLH ITPGTAYQSF EQ..... DTFVE LYGNNAAAES
bcl-x_I VVNLF RD GVNWGRIVAF FSFGGALCVE

bcl-x_L SVDKEMQVLV SRIAAMATY LNDHLEPWIQ ENGGWDTFVE LYGNNAAAES
bcl-x_S DTFVE LYGNNAAAES
bcl-x_I SVDKEMQVLV SRIAAMATY LNDHLEPWIQ ENGGW

bcl-x_L RKGQERFNRW FLTGMTVAGV VLLGSLFSRK
bcl-x_S RKGQERFNRW FLTGMTVAGV VLLGSLFSRK

* ----- *

Fig. 4 c

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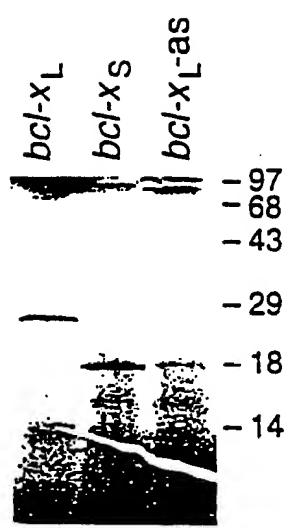


Fig. 5

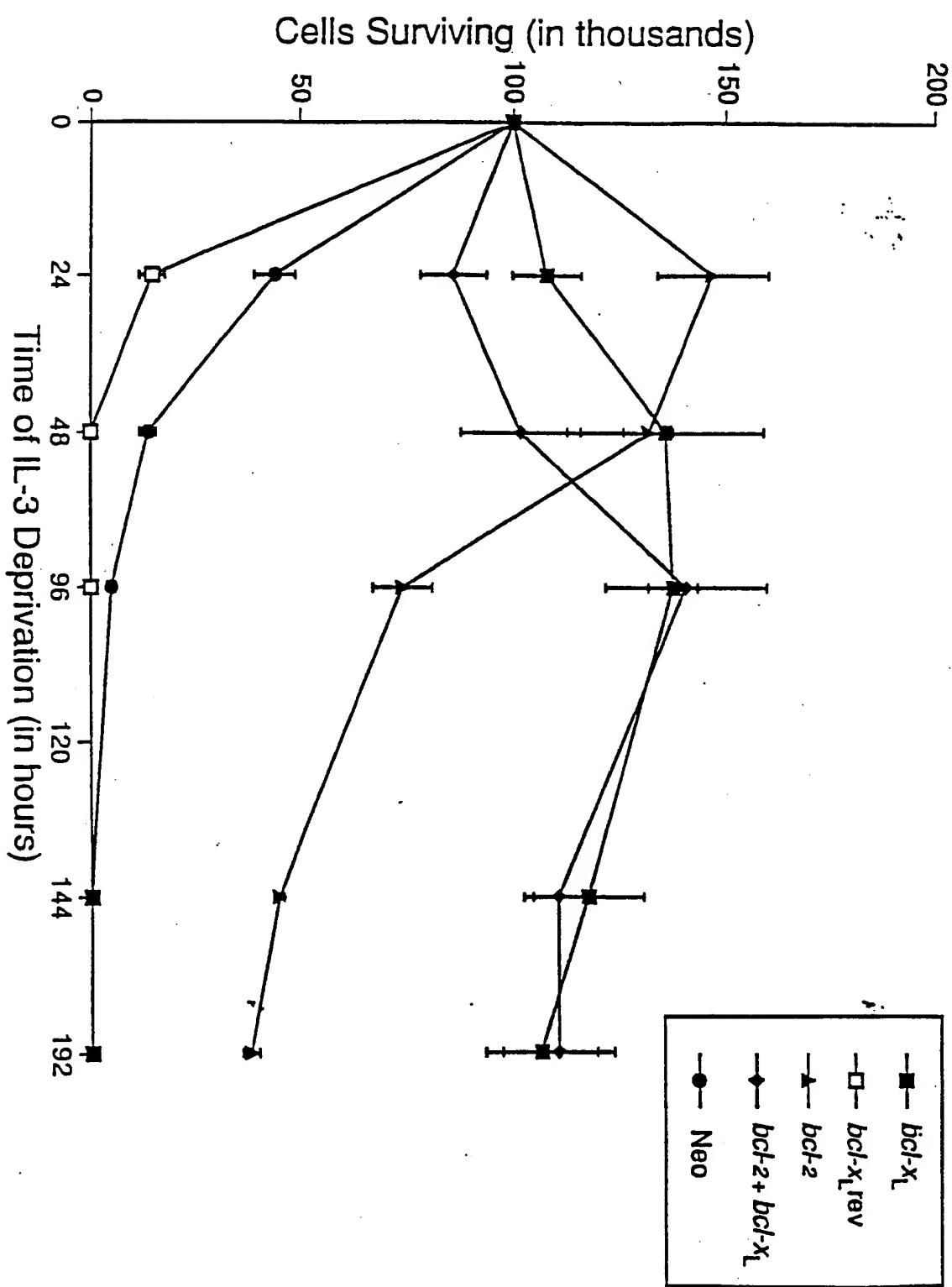
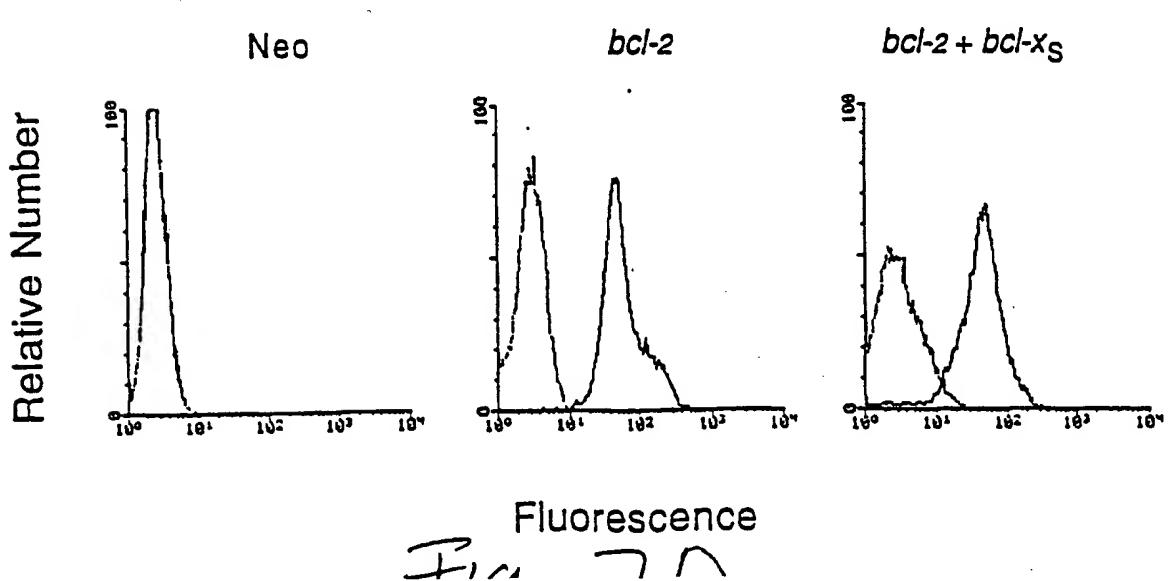
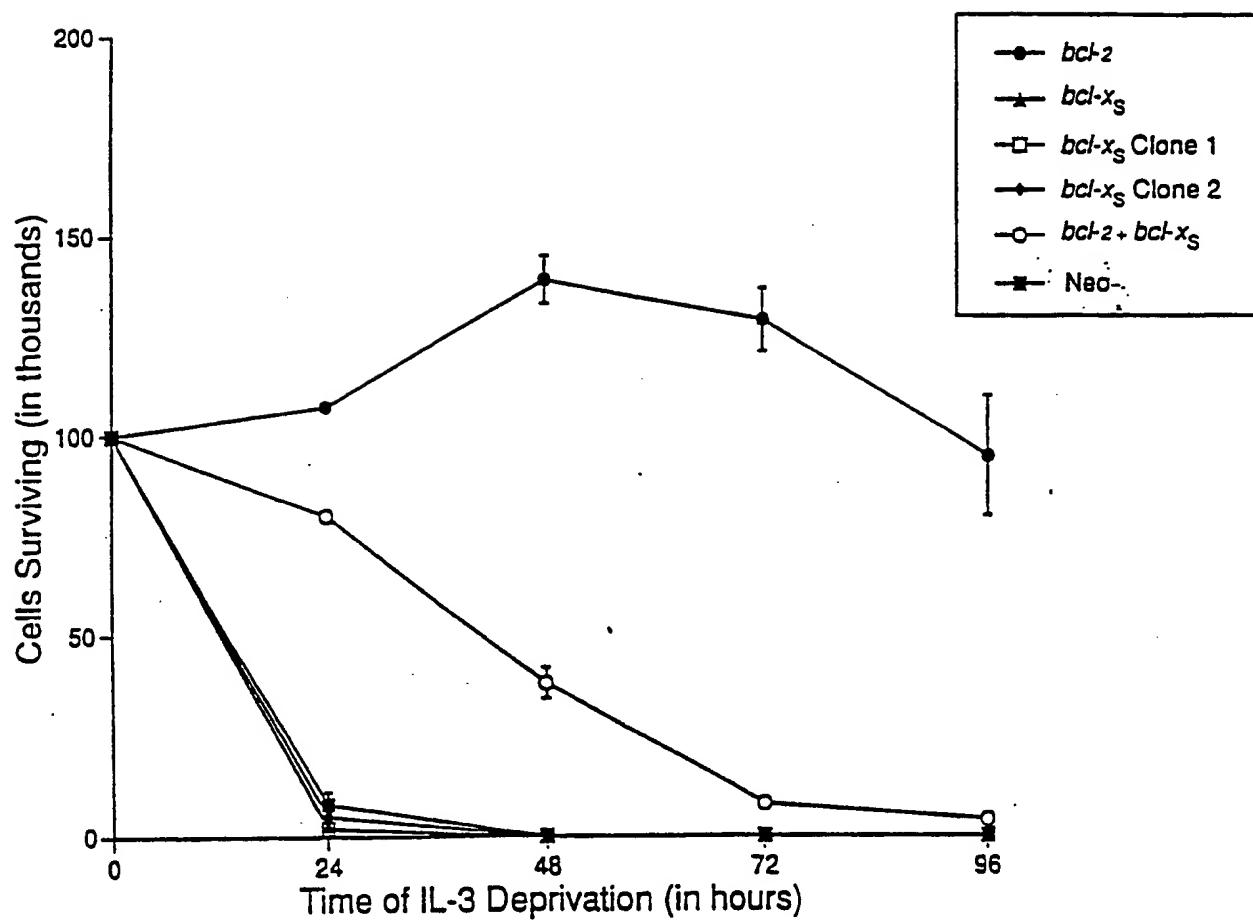
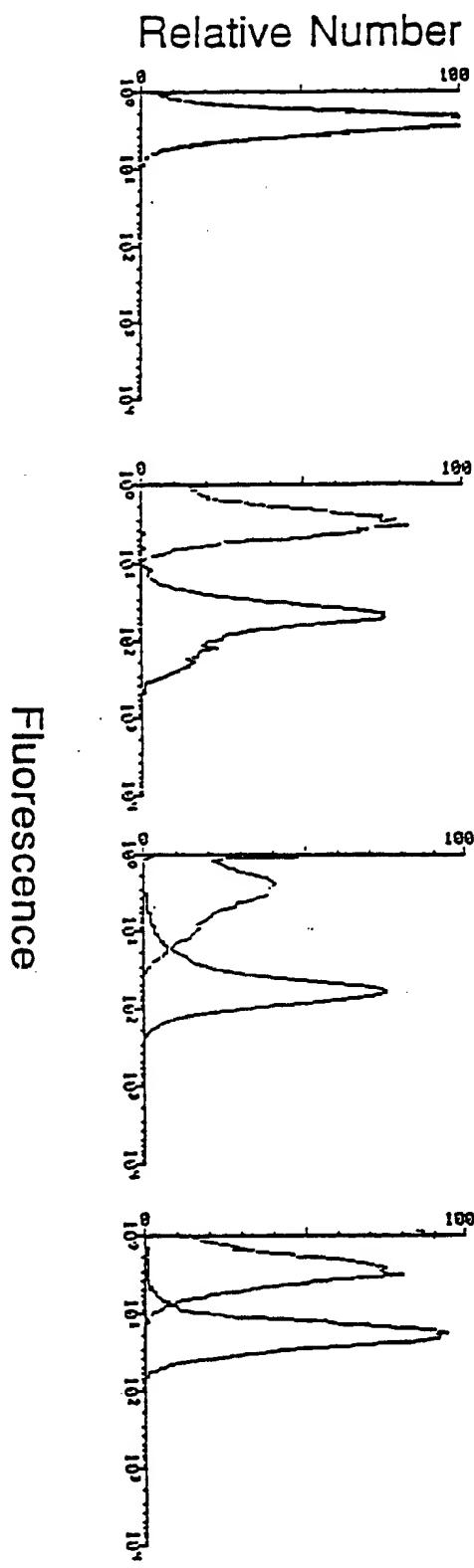


Fig. 6

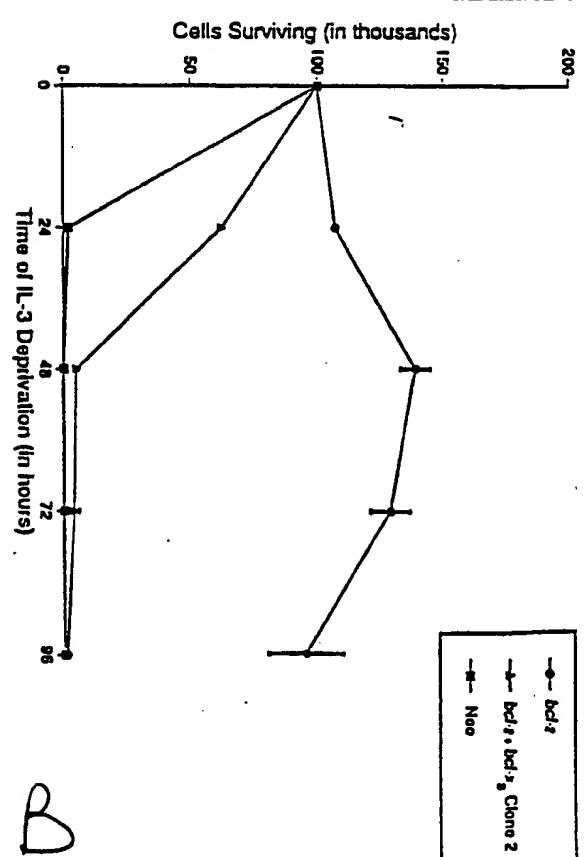
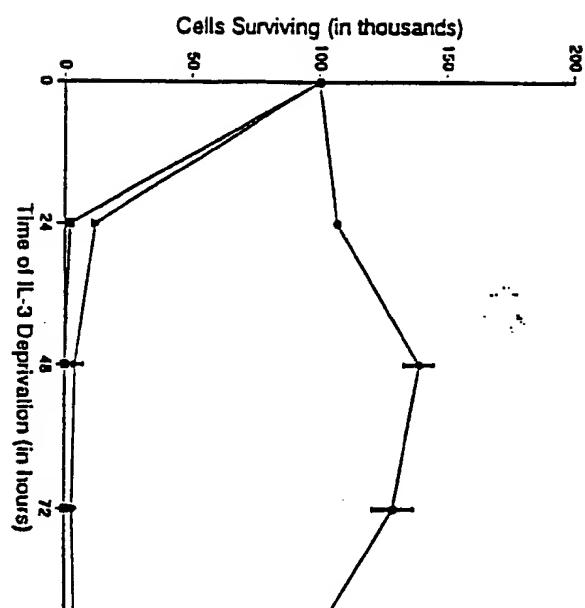
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A
7
B
a



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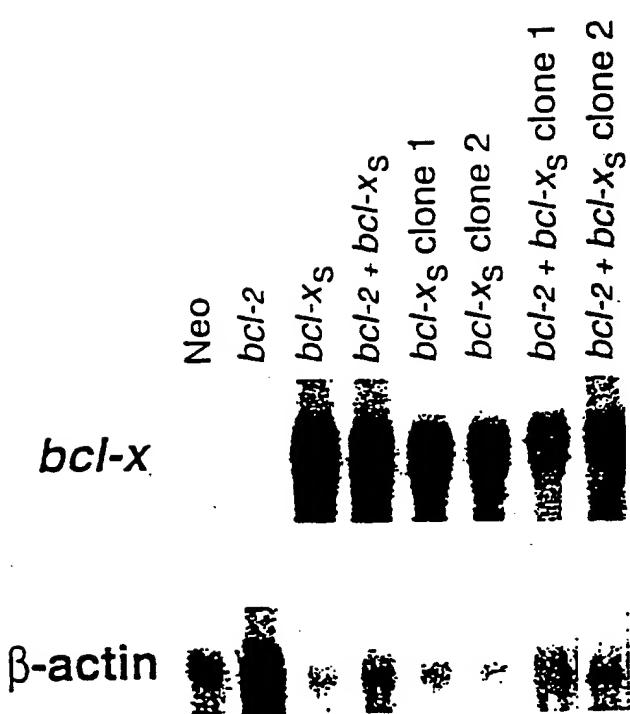
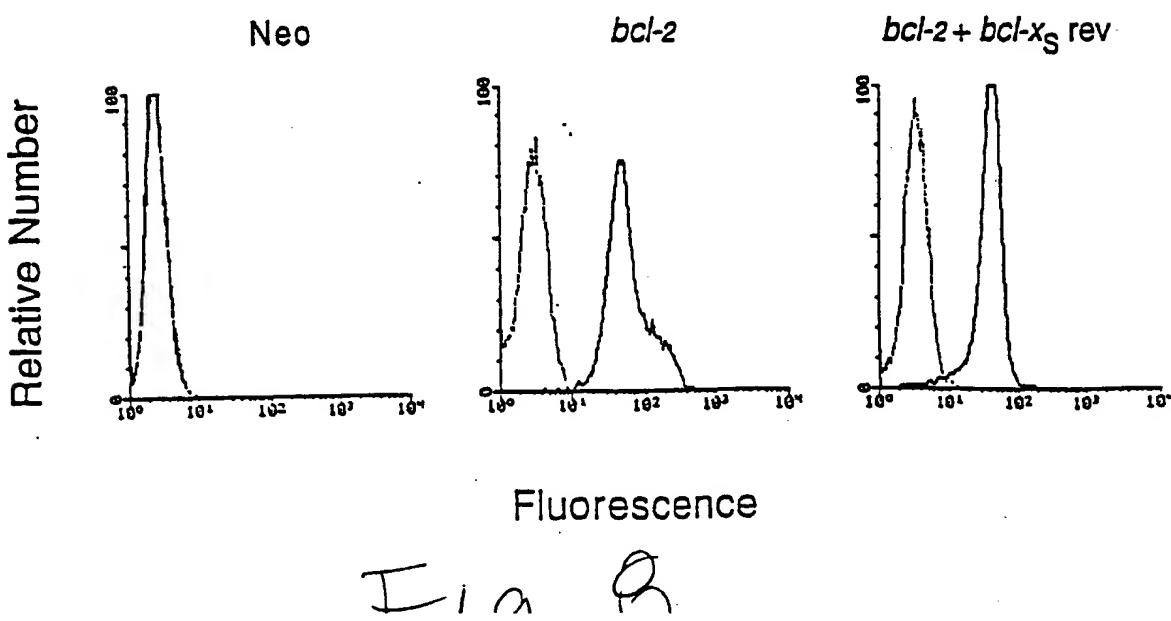
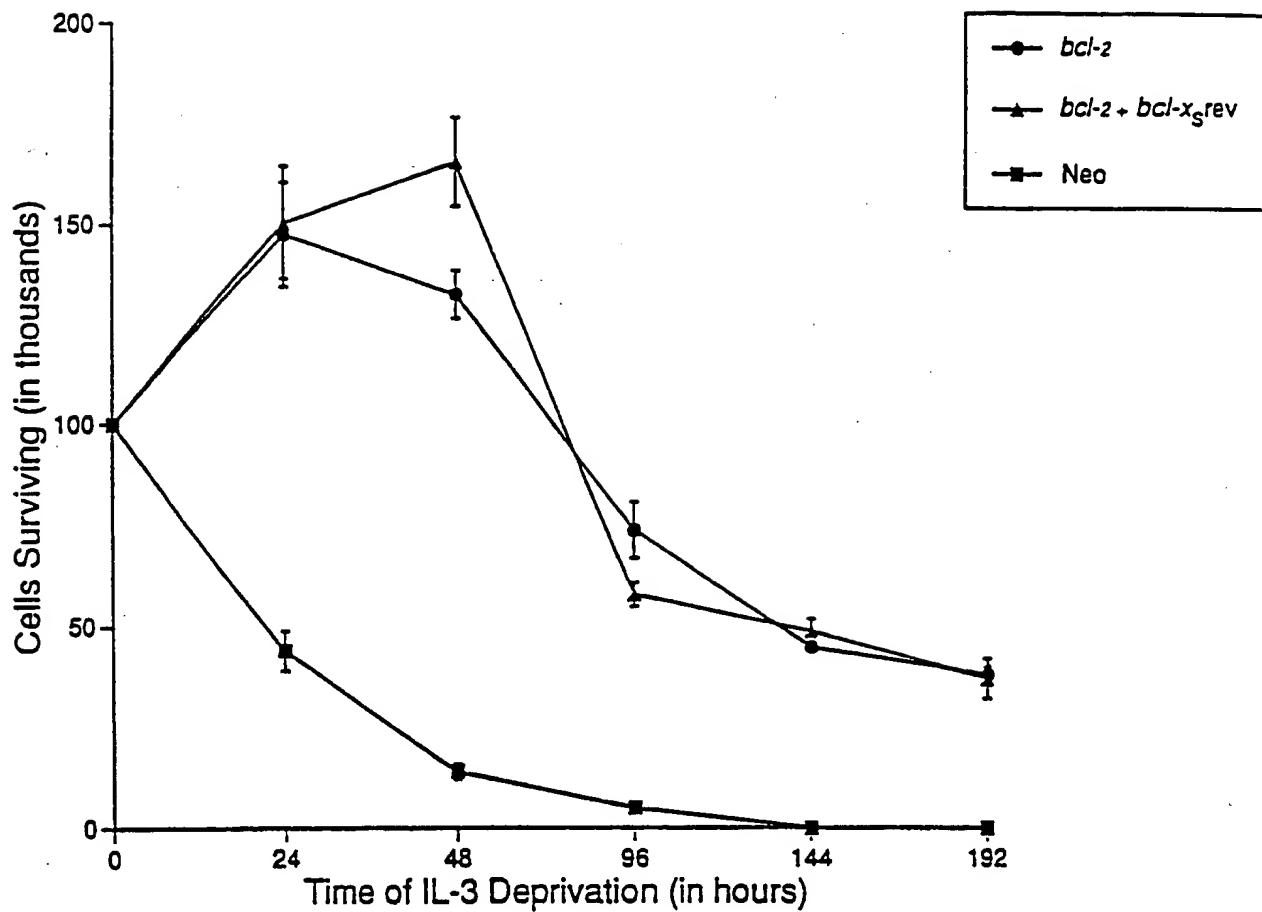


Fig. 7C

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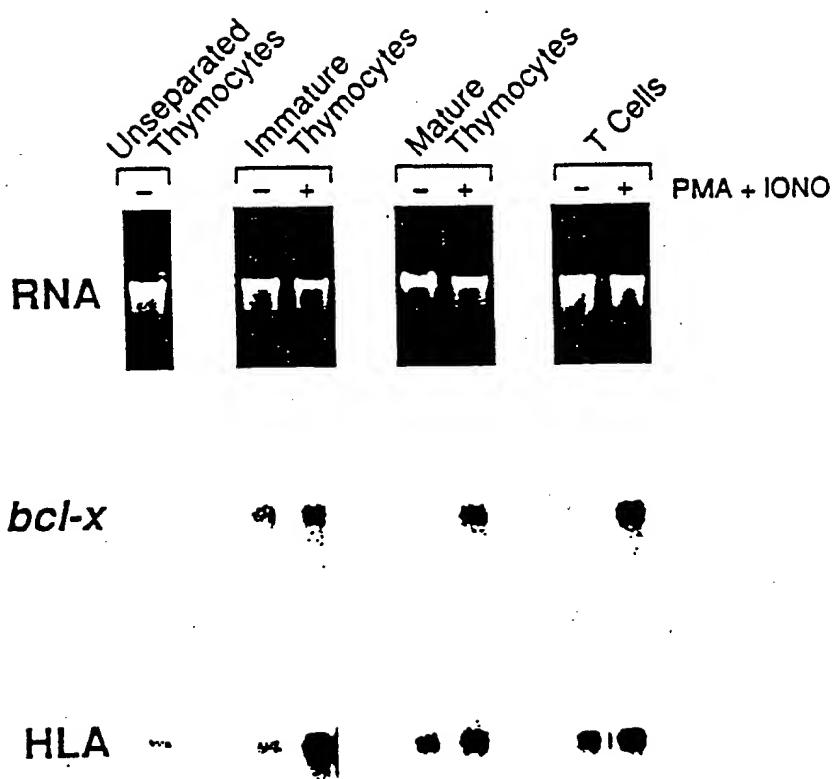
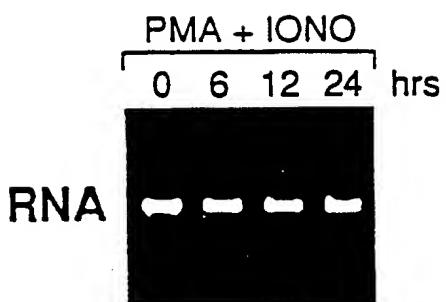


Fig. 9

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bcl-x ■ ■ ■

bcl-2 ■ ■

HLA ■ ■ ■ ■

Fig. 10

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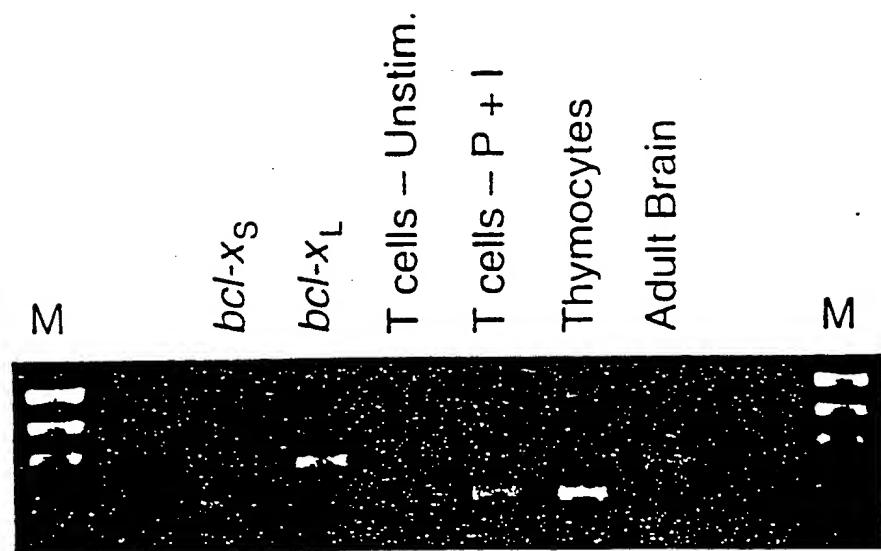


Fig. 11 A

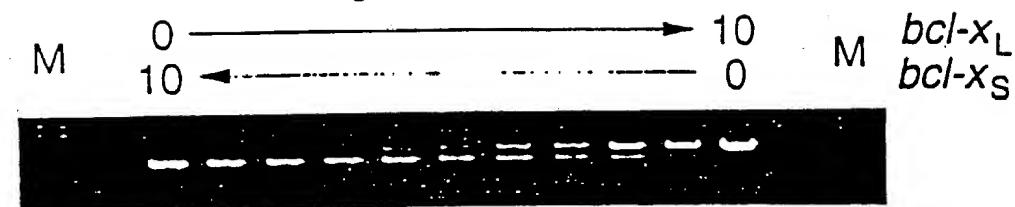


Fig. 11 B

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5	C12N15/12	C12N15/70	C07K13/00	C12N1/21	C12P21/08
	G01N33/53	C12Q1/68	C12Q1/00	A61K48/00	A61K37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K C12N C12Q A61K G01N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.90, no.8, 15 April 1993, WASHINGTON US pages 3516 - 3520</p> <p>KAREN M. KOZOPAS ET AL. 'MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2'</p> <p>see abstract</p> <p>see page 3516, right column, paragraph 2</p> <p>see page 3517, left column, paragraph 4</p> <p>see page 3518, right column, last paragraph - page 3519, left column, paragraph 1</p> <p>see page 3520, left column, last paragraph</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/-</p>	1,2



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

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Date of the actual completion of the international search

29 September 1994

Date of mailing of the international search report

19.10.94

Name and mailing address of the ISA

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Authorized officer

Montero Lopez, B

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, vol.349, no.6310, 14 February 1991, LONDON GB pages 612 - 614 CHRISTOPHER D. GREGORY ET AL. 'Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis' see abstract ----	
P,X	CELL, vol.74, 27 August 1993, CAMBRIDGE, MA US pages 597 - 608 LAWRENCE H. BOISE ET AL. 'Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death' see summary see page 598, left column, paragraph 2 - page 599, right column, paragraph 1 see page 600, left column, paragraph 1 - page 601, left column, paragraph 1 see page 603, right column, paragraph 2 - page 604, left column, paragraph 1 see page 604, right column, paragraph 2 - page 605, left column, paragraph 1 see page 606, left column, paragraph 2 ----	1-10,12, 14,19, 20,24, 25,34, 41,43
P,X	CELL, vol.74, 27 August 1993, CAMBRIDGE, MA US pages 609 - 619 ZOLTAN N. OLTVAI ET AL. 'Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death' see summary see page 609, right column, last paragraph see page 610, right column, last paragraph - page 611, right column, paragraph 1 see page 613, left column, paragraph 2 - right column, paragraph 2 ----	1,2,7, 10-12, 14,34,41
P,X	DATABASE WPI Section Ch, Week 9404, Derwent Publications Ltd., London, GB; Class B04, AN 94-030912 HONJO Y 'Mammalian programmed cell death protein PD-1 - from human and mouse and PD-1 genes, used for isolation of other PD-1 genes' & JP,A,5 336 973 (ONO PHARM CO LTD) 21 December 1993 see abstract -----	1,2,7, 10-12,14
1		

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 24-26, 33-35, 39, 40, 45, 46 partially and 27-32, 36, 37, 41-44 totally are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.

2. Claims Nos.:

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.